GENERATION OF CYTOTOXIC T LYMPHOCYTES

IN VITRO

A thesis submitted in fulfilment of
the requirements for the degree
of

DOCTOR OF PHILOSOPHY

at the

Australian National University

by

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JANUARY 1979

STATEMENT

The experiments described in Chapter 4 were carried out in conjunction with Drs. D.W. Talmage and K.J. Lafferty and those in Chapter 5 in conjunction with Dr. I.S. Misko. With these exceptions, the experiments reported in this thesis were performed by myself.

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ACKNOWLEDGEMENTS

The work described in this thesis was carried out in the Department of Immunology, John Curtin School of Medical Research, Australian National University, and I thank Professor Bede Morris for the opportunity to study in his department.

Thanks must also go to my supervisor, Dr. Kevin Lafferty, for his constant help and critical advice throughout the duration of the project, and the preparation of this thesis.

I would also like to thank the members of the Department of Immunology and Microbiology, J.C.S.M.R., for their helpful suggestions and discussion.

ABSTRACT

The work presented here represents an attempt to characterize the response of T cells to transplantation antigens *in vitro* with the view to formulating a useful theoretical model of the system.

We believe that a number of basic characteristics of these responses can be defined. A crucial observation is that histocompatibility antigen alone is not immunogenic for T cells. Hence, at least two signals are required for T cell activation; antigen (signal 1) and a further inductive signal (signal 2) which is provided by a special class of stimulator cell within the reticulo-endothelial system. Evidence is presented that both signals 1 and 2 can be provided by the same cell indicating that a distinct class of non-cytotoxic helper T cell is not required for the generation of such responses.

A further characteristic of T cell responses *in vitro* is their phylogenetic restriction. T cells will respond only to stimulator cells from allogeneic or closely related xenogeneic animals. No responses to unrelated xenogeneic cells occur. In Chapter 5 evidence is presented suggesting that this phylogenetic restriction is due solely to the phylogenetic specificity of the inductive second signal and not to a lack of precursor cells potentially reactive to xenoantigen.

In the final chapter a two cell interaction model for T cell activation is presented.

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CHAPTER ONE

IMMUNE RESPONSES TO TRANSPLANTATION ANTIGENS; A LITERATURE REVIEW.

1.1 HISTORICAL BACKGROUND

In 1911 Carrel described the successful autologous transplantation of a kidney in the dog (Carrel, 1911). The success of this experiment led him to conclude that 'from a purely surgical standpoint, the grafting of organs (was) a real possibility'. In accepting the Nobel Prize the following year, in fact, he went so far as to claim that from the technical point of view the problem of organ transplantation (had) been solved' (Nuboer, 1969). And so the surgical art of tissue and organ transplantation received world-wide recognition as a major clinical breakthrough.

Unfortunately for the transplant surgeon, with the overcoming of technical barriers to transplantation an unknown biological factor still frustrated the technical successes in *all* cases of allo- and xenotransplantation. The cessation of function in allotransplanted organs was almost always noted between five and seven days after transplantation, and Williamson (1923) stressed the marked distinction between the reactions of allogeneic and autologous organ transplants. Only on the rare occasions of organ transplantation between identical twins, such as the first clinical example described by Merrill *et al* (1956), were organ transplants between humans a complete success.

From the time graft rejection was unquestionably recognized as an immune phenomenon (Gorer, 1937; Medawar, 1945, 1954) this field of immunology has both stimulated an ever-increasing volume of research and precipitated many new concepts in our

understanding of the immune system. Following the discoveries of Gorer and Medawar, many workers sought and found evidence for a humoral antibody response to normal and neoplastic allografts in experimental animals (Gorer, 1942, 1947; Mitchison and Dube, 1955; Bollag, 1956), for at the time 'cell-mediated immunity' was not a well characterised phenomenon.

Nevertheless, it became apparent that although antibodies were easily demonstrable following allotransplantation they could not be implicated as active mechanisms of graft rejection. Many workers found that transplantation immunity could not be transferred via serum. Billingham and Brent (1956) were unable to transfer any immunity to skin grafts via hyperimmune serum using multiple variations in serum dosage, regimen of administration, and route of injection. They were forced to conclude that serum antibodies were *not* the agents of graft rejection. Mitchison and Dube (1955) in an elegant series of experiments were able to show that the antibody response to allogeneic tissue was not related to graft rejection and clearly characterised the 'cell-mediated' nature of the response. They found that viable, lyophilised or frozen tumour tissue when injected into allogeneic recipients caused an antibody response in the draining lymph nodes. Lymph node cells from *all* recipients could be used to transfer that antibody response to secondary recipients. However, only lymph node cells exposed to viable tumour cells could be used to transfer graft rejection reactivity. In other words, lyophilised or frozen allogeneic tissue could stimulate an antibody but not a graft rejection response in the draining lymph node. Cells transferred

to a secondary host from these lymph nodes were not only unable to increase anti-graft reactivity in the recipients but actually afforded some protection to subsequent grafts. Also, graft rejection reactivity could only be transferred via lymph node cells exposed to viable tumour cells, never by serum or antibody reactive cells.

Some years later Miller (1962) demonstrated the essential role of the thymus in transplantation immunity. At the same time others (Rosenau and Moon, 1961; Vainio *et al.*, 1964) discovered, in recipients of allogeneic tissue, lymphoid cells which were able to lyse target cells of the appropriate donor type. Cerottini *et al* (1971) used a system of injecting spleen cells into lethally-irradiated recipients to show that the responding cytotoxic cells bore the Thy-1 antigen (then known as the θ antigen) which was first detected on thymic and nervous tissue of the AKR mouse (Reif and Allen, 1964) and later shown to be a cell surface marker for thymus-derived lymphocytes in mice (Raff, 1969).

In this way a concept of cytotoxic T cell responses playing an essential role in graft rejection evolved, a concept which was compatible with Miller's observations of graft rejection being a thymus-dependent response. Häyry and Defendi (1970) demonstrated that cytotoxic cells could be generated in culture by stimulation with allogeneic cells and referred to this mixed lymphocyte culture (MLC) system as an *in vitro* model for allograft rejection. Both the proliferating (Mosier and Cantor, 1971; Tyan and Ness, 1972) and cytotoxic cells (Häyry *et al.*, 1972) responding in MLC were subsequently shown to be Thy-1-positive T cells.

At this stage our knowledge with respect to cytotoxic T cell function in graft rejection must be close to that of antibody in the early 1950's. We know they are generated during the response and we know that they exhibit activity against graft type cells *in vitro* but that does not necessarily imply that they are the active agents involved in graft rejection. It has become quite apparent that the response to allogeneic tissue *in vivo* involved a great variety of lymphoid cells; T cells, B cells, non-specific inflammatory cells such as those of the myeloid class, and K cells, which are responsible for antibody-dependent lysis. K cells would appear to occur in large numbers during allograft rejection (Forman and Britton, 1973; Häyry and Roberts, 1977) but are as yet an unclearly defined mediator of cell-mediated immunity. Nevertheless, Pearson (1978) has suggested that K cells may comprise a major aspect of cell-mediated immunity since they appear to be capable of carrying out most effector functions so far attributed to cytotoxic T cells.

Which cells play an active role in graft rejection or are, indeed, even required for graft rejection is as yet unknown.

1.2 GENETIC BASIS OF GRAFT REJECTION

Our understanding of the genetic control of allograft rejection is attributable in the main part to the early work of Snell and Gorer. Snell (1948) defined antigens responsible for tissue compatibility as histocompatibility antigens and the genes coding for these structures as histocompatibility genes (H genes). Using several newly produced congenic strains

of mice he and Gorer (Gorer *et al.*, 1948) identified a major genetic locus responsible for resistance to allogeneic tumour. This locus, which they designated H-2, was linked to the gene for 'fused tail (Fu)' assigned previously to linkage group IX. Despite the discovery that genetic differences at regions other than H-2 could lead to the rejection of skin grafts (Counce *et al.*, 1956) this phenomenon was never as strong as that seen with differences at H-2. Hence, the H-2 genetic complex has become known as the 'major histocompatibility complex' (MHC) of the mouse. A similar genetic complex has been characterized for all mammalian species so far investigated. In man the MHC is called H-LA.

With the development of efficient serological techniques for the detection of antigens controlled by the H-2 locus (Gorer and Mikulska, 1954; Gorer and O'Gorman, 1956) the complexity of the murine MHC was revealed. This was further realized by the discovery that certain genes within the MHC controlled the level of humoral antibody responses to certain synthetic polypeptides (McDevitt *et al.*, 1972). These genes, which were called 'Ir' genes, gave the first indication that the MHC represented a general immunological control structure for both humoral and cell-mediated responses.

Our present picture of the murine MHC involves a series of genetic regions each with a distinct function but united in that they all have some immunological importance (Klein, 1975a). Three of these regions have direct relevance to the studies in this thesis with respect to cytotoxic T cell activation. Two regions (H-2K and H-2D) code for the antigens

first described serologically by Snell and Gorer. These antigens are found on glycoprotein molecules consisting of two chains (Silver, 1977), one of 45,000 daltons coded for by the H-2K or H-2D region of the MHC and one of 12,000 daltons, which appears to be identical to β_2-microglobulin (Silver and Hood, 1976), an immunoglobulin-like molecule. The 45,000 dalton chain demonstrates a marked homology with similar glycoproteins coded for by the A sub-region of the human MHC (H-LA) (Klein, 1977). The β_2-microglobulin-like chain is not coded for by the MHC and is non-covalently associated with both murine and human histocompatibility antigens (Goodfellow *et al.*, 1975; Silver, 1977).

Antigens coded for by the I-region of the murine MHC are present on a distinct class of glycoprotein molecule but which have a similar basic structure to the H-2K/D-coded molecules. I-region-associated (Ia) antigens occur on a glycoprotein structure which also consists of two non-covalently linked chains, a heavy chain (34-35,000 daltons) coded within the H-2I subregion and a light chain (28-29,000 daltons) coded by a genetic region not linked to the MHC. Again there is a distinct homology between similar molecules coded for by murine (H-2I) and human (HLA-D) subregions of the MHC. The tissue distribution of H-2I associated antigens in the mouse and HLA-D associated antigens in man is also strikingly similar (Silver *et al.*, 1977; McMillan *et al.*, 1977).

In summary, immune responses in both man and mouse are strictly controlled by a genetic region, designated as the MHC. Two distinct regions affecting immune responses can be identified

in both species. The H-2K and D (H-2K/D) regions of the murine MHC code for histocompatibility antigens present in varying amounts on most cells of the body. This region corresponds to the A and B subregions of the human MHC (H-LA). The H-2I region of the MHC controls humoral immune responses and codes for Ia antigens which exhibit a limited distribution being found predominantly on some classes of blood cells. All subregions described above are involved in T cell responses *in vitro*.

1.3 ANTIGENIC REQUIREMENTS FOR CELL-MEDIATED RESPONSES TO ALLOGENEIC TISSUE *IN VIVO*

Considerable controversy has surrounded the role of antigen in the induction of cell-mediated allograft responses. Early reports pointed out the enigmatic properties of transplantation antigens presented *in vivo* on anything but viable lymphoid (white blood cell) - derived cells. The experiments of Mitchison and Dube (1955) described above suggested that non-viable tissue although antigenic and capable of inducing a humoral immune response, was not immunogenic for the cell-mediated graft rejection response. Billingham *et al.*, (1956) found that neither killed leucocytes nor non-lymphoid cells such as erythrocytes could induce specific transplantation immunity in newborn chickens. Later studies by Lafferty and Jones (1969) and Elkins (1971) indicated that allograft reactions of the graft-versus-host type also involved antigen presented in particular on cells of haemotogenous origin. Observations such as these were not altogether compatible with the concept of lymphocyte activation held at that time. Medawar (1963) referred to antigen, with respect to both T and B cell responses,

as an inducer molecule, similar to embryonic inducers, which directly stimulated the responding cell to proceed through its particular differentiation pathway. Why the same antigens present on different classes of cells demonstrated widely varying degrees of immunogenicity was, at that time, difficult to explain and often ignored completely.

The 'Medawar' concept was not threatened to any great extent by the numerous studies implying that leucocytes passively carried across with a graft were the prime immunizing agents leading to the ultimate rejection of the graft (Billingham, 1971). Such observations could be accounted for by the proposition that the leucocytes, being a highly motile cell population, were presented much more readily to the host's immune system, either in the draining lymph nodes or spleen, than were the predominantly sedentary parenchymal cells of the graft.

Perhaps the best evidence against this hypothesis lies in some of the earliest experiments. Mitchison and Dube (1955) demonstrated quite clearly that a draining lymph node could be exposed to allogeneic tissue and generate a humoral response without generating a cell-mediated allograft response. Lafferty *et al.*, (1976) found that thyroid lobes of mice, after 26 days of organ culture, were not rejected if transplanted beneath the kidney capsule of allogeneic mice. This has been attributed to the loss of passenger leucocytes during culture. Lobes treated in the same way, however, were able to stimulate a strong response in the popliteal lymph nodes following transplantation to the footpad, but this response did not include the

cytotoxic T cell response observed when untreated thyroid tissue was transplanted to the same site. These experiments demonstrate that culturing for 26 days destroys the immunogenicity of thyroid tissue with respect to the graft rejection response. However, the tissue is still immunogenic for the draining lymph nodes when transplanted to the footpad, indicating that graft antigens are still presented to the draining node despite the loss of passenger leucocytes during culture.

1.4 ACTIVATION OF T CELLS BY ALLOGENEIC ANTIGEN *IN VITRO*

Despite the still questionable role of T cells in the graft rejection response a great deal of attention has been afforded the activation of T cells by allogeneic antigen *in vitro*. This may be partly attributable to the fact that murine and human T cells have proven so amenable to culture. Nevertheless, the ease with which T cell responses can be observed in culture have allowed their detailed study in a system devoid of some of the complexities of the *in vivo* situation. Although there are, inherently, many risks involved with the study of any biological system isolated from its true physiological *milieu*, such studies afford us the opportunity to gain detailed knowledge of the activation of one class of specific immune cell.

The development of *in vitro* techniques for the study of T cell responses to histocompatibility antigens has provided considerable evidence against the concept of antigen providing the sole inductive stimulus for these responses. Evidence accumulated over the last decade has suggested that responses

of T cells to histocompatibility antigens *in vitro* are subject to the same limitations as the allograft response *in vivo*. Only histocompatibility antigens presented on viable lymphoid cells, it would seem, are immunogenic.

Lymphoid cells whose metabolic activity has been disrupted by a variety of techniques lose their capacity to stimulate in MLC. Ultraviolet (UV) radiation (Lindahl-Kiessling and Säfwenberg, 1972; Lafferty *et al.*, 1974; Schendel and Bach, 1975) or high levels of γ-irradiation followed by 24 hours of tissue culture (Lafferty and Jones, 1969) destroys the capacity of leucocytes to stimulate allogeneic T cells in MLC. Such cells, however, retain their histocompatibility antigens intact as indicated by serological techniques (Lindahl-Kiessling and Säfwenberg, 1972; Lafferty *et al.*, 1974), and by their capacity to stimulate a secondary cytotoxic T cell response (Wagner and Röllinghoff, 1976) or a primary response under suitable culture conditions (Schendel and Bach, 1975; Lafferty *et al.*, 1974). Schellekens and Eijsvoogel (1970) have investigated in some detail the effects of a number of metabolic inhibitors on the stimulatory capacity of human peripheral blood leucocytes (PBL) in allogeneic MLC. They found that PBL treated with heat (45°C for 60 minutes) were unable to stimulate in MLC despite the fact that their H-LA antigens were intact. This has also been found in the murine MLC system (Schendel and Bach, 1974). Furthermore, treatment of cells with iodo-acetate at concentrations sufficient to completely shut down lymphocyte glycolysis, led to a marked reduction in stimulatory capacity. Freezing and thawing consistently led to a complete loss of stimulatory capacity. Treatment with KCN, however, which

interferes with mitochondrial function via the cytochromeoxidase reaction, had no effect on the stimulatory capacity of PBL. This suggests that all energy or metabolic requirements for stimulation can be completely covered by glycolysis.

Considerable evidence has also accumulated with respect to the role lymphoid cells play in the stimulation of T cell responses in MLC. It has been reported in a variety of systems, including both human and murine MLC, that non-lymphoid cell populations such as thyroid, HeLa, human melanoma, and other non-lymphoid neoplastic cell lines, fibroblasts and polymorphonuclear leucocytes as well as solubilised cell surface antigens and microsomal cell fractions do not stimulate in allogeneic MLC (Pulvertaft and Pulvertaft, 1967; Hardy and Ling, 1969; Gutterman *et al.*, 1972; Han, 1972; Wagner and Wyss, 1973; Greineder and Rosenthal, 1975). The capacity of dissociated skin cells to stimulate in MLC has not been quite so clear-cut. Many workers have independently reported that skin epithelial cell preparations stimulate allogeneic lymphocytes in MLC (Gillette *et al.*, 1972; Lewis and Miller, 1972; Hirschberg *et al.*, 1973, 1975; Steinmuller and Wunderlich, 1976). Lane and co-workers, on the other hand, have reported that skin epithelium under some conditions will not stimulate in MLC, and that its capacity to stimulate depends both on the way it is prepared (Lane and Ling, 1973) and maturity of the donor animal (Lane *et al.*, 1975). When interpreting experiments of this type it must be remembered that, as pointed out by Cochrum *et al* (1971), stimulation by skin epithelium may be attributed to the small number of contaminating leucocytes found in any such preparation. Only neoplastic cell lines

which have been cloned and passaged in culture for some time are likely to be entirely free of such contaminating cells.

A number of workers have claimed that MLC reactions do not occur between xenogeneic combinations of cells (Wilson and Nowell, 1970; Wilson and Fox, 1971; Lafferty and Jones, 1969; Greineder and Rosenthal, 1975). This aspect of MLC reactivity has been particularly controversial, since a great number of workers have reported just the opposite (Asantila *et al.*, 1974; Nielson, 1972; Shons *et al.*, 1973; Widmer and Bach, 1972; Lindahl and Bach, 1975, 1976; Peck *et al.*, 1976). Klein (1975b) referred to any suggestion that MLC reactivity between xenogeneic combinations of cells was either nonexistant or weak as a 'myth'. Perhaps one outstanding point with respect to xenogeneic MLC reactivity is that the suggestion that such reactions are weak conflicts with the basic concept of MLC reactivity representing an *in vitro* model for graft rejection. It has been shown that grafts between different species are not only rejected, but in an accelerated fashion (Russell and Monaco, 1965; Steinmuller, 1961; Egdahl *et al.*, 1958). An allogeneic skin graft on the rat survives for an average of 9-10 days. A rabbit skin graft to the same recipient is completely rejected within 5-6 days. Superficially, it would seem, xenograft rejection is simply a more intense form of allograft rejection.

There has, for some time, existed evidence that allograft and xenograft rejection may involve quite distinct mechanisms. Murphy (1914) observed that the chicken embryo was capable of rejecting a rat sarcoma in the absence of the mononuclear cell

infiltration which is a characteristic of homograft reactions, and it has become a general observation that xenograft reactions exhibit a less extensive cellular infiltration than do allograft reactions (Russell and Monaco, 1965). Another distinguishing feature of xenograft reactions is that it is possible in some circumstances to transfer immunity with serum (Hasková *et al.*, 1962), whereas immunity to allografts cannot be transferred via serum under any circumstances. One group of workers (Weaver *et al.*, 1955; Algire *et al.*, 1957) found that xenogeneic (rat), but not allogeneic tissue, was rejected if it was enclosed in 0.45μ Millipore chambers and transplanted to pre-immunized recipient mice. The allogeneic grafts were rejected only if enclosed in chambers which permitted the entry of cells.

These studies suggest that a humoral immune response can in some circumstances cause the rejection of a xenograft. That antibodies play a significant role in xenograft reactions is compatible with the observations of Lambert and Hanes (1911) that certain tumours would not grow in the serum of xenogeneic innoculated animals, and that antibody-dependent rather than T cell-mediated cytotoxicity is more apparent in xenograft than in allograft systems (Cerottini and Brunner, 1974).

Despite these observations the question of xenograft reactivity has received scant attention in recent years. Perhaps this is partly because observations such as these were entirely incompatible with the generally held concepts at the time. Similarly, the question of xenogeneic reactivity in MLC has remained unresolved to this day, and no real attempt to resolve it has yet been made. Lafferty *et al.*, (1972) have

not only observed a lack of reactivity between xenogeneic tissues in GVH reactions but have suggested that this phenomenon has important theoretical implications. In Chapter 5 are described a number of experiments, carried out in our laboratory, which were designed to re-examine the question of xenogeneic reactivity in MLC. In this chapter, I will discuss evidence for our conclusion that MLC responses between phylogenetically unrelated cells do not behave comparably to their allogeneic counterparts, and I will discuss further the theoretical implications of these findings.

1.5 MODELS FOR T CELL ACTIVATION

Two basic models have been proposed to account for the fact that only histocompatibility antigens on viable lymphoid cells have the capacity to stimulate in MLC. The first, proposed independently by a number of workers (Hardy and Ling, 1969; Schellekens and Eijsvoogel, 1970; Lindahl-Kiessling and Säfwenberg, 1972; Lafferty *et al.*, 1972) postulates that T cells do not repond to histocompatibility antigens alone but require, in addition to antigen, a factor which must be passed from the stimulating to the responding cell. The stimulating cell must, consequently, be both metabolically intact and of the particular class of cell, probably of haematogenous origin, capable of providing the appropriate inductive signal. This model also provides a possible explanation for any lack of responsiveness between xenogeneic cells for any species specificity of the inductive factor would lead to an apparent species specificity of the T cell responses themselves.

This model has not received a great deal of attention over recent years and has given way in the literature predominantly to an alternative model developed over a number of years by F.H. Bach and co-workers. This model proposes, similarly, that histocompatibility antigens (H-2K/D-coded SD antigens) alone are not sufficient to induce T cell activation. They have proposed that collaboration between two classes of T cells is required for the ultimate expression of cytotoxic T cell activity. Non-cytotoxic helper T cells, responding to H-2I region-coded determinants (HLA-D in man) are required to allow the complete differentiation of cytotoxic T cells, which themselves respond predominantly to SD (HLA-A/B in man) antigens.

This model is based on a number of observations with respect to the induction of T cell responses to histocompatibility antigens on killed stimulator cells or to foreign H-2K/D coded SD antigens on congenic strains of mice. Schendel and Bach (1974) found that the non-responsiveness in allogeneic MLC to heat-killed stimulating cells could be overcome by adding a population of third party, H-2I region disparate cells to the culture system. The presence of a population of intact cells, which differed from the responding cells at the H-2I region of the MHC, was able to induce, in the responding cells, a cytotoxic response to the SD antigens on the heat-killed stimulating cells. Very little cytotoxic activity was observed against the H-2I region-disparate stimulating cells. Also, Bach and co-workers (Bach *et al.*, 1972, 1973, 1976; Alter *et al.*, 1973; Schendel *et al.*, 1973) and others (Abbasi *et al.*, 1973; Wagner *et al.*, 1975) often observed a marked synergy between the stimulating capacities of H-2K/D coded (SD) antigens and

H-2I coded determinants with respect to the generation of anti-SD cytotoxic responses. Again, little cytotoxic activity was observed against target cells of the appropriate H-2I genotype. A similar effect was observed in human *in vitro* cytotoxic responses (Eijsvoogel *et al.*, 1973).

These observations led Bach (1973) to propose a model for cytotoxic T cell activation involving the interaction of two distinct classes of T cells, cytotoxic (T_C) and helper (T_H) cells. The former, he proposed, were activated against SD antigens (H-2K/D-coded) and the latter were stimulated by lymphocyte-defined, or LD (H-2I-coded), determinants. This model was later re-expressed in a more elaborate form (Bach *et al.*, 1976, 1977), proposing now that SD antigens could drive the cytotoxic precursor cells through part of their differentiation pathway to become 'poised' cytotoxic precursor cells which were then receptive to the effect of T_H cells either directly or via some T_H-elaborated helper factor.

The two models described above represent the two main schools of thought with respect to the activation of cytotoxic T cells to allogeneic antigen *in vitro*. Their predominant difference rests in the fact that the former sees the stimulating cell as playing an *active* role in the activation event. A second inductive signal, perhaps soluble, must be passed from the metabolically intact stimulator cell to the responding cell. The latter model proposed by Bach, on the other hand, sees the stimulating cell in an entirely *passive* role. All that is required to achieve T_C activation is the coincidental occurrence of SD antigens and antigenic determinants (mainly coded by the H-2I genetic region of the mouse) which activate, directly, helper T cells.

Alter *et al.*, (1973) and Schendel and Bach (1974) reported that spleen cells responding to congenic stimulator cells which differed at the H-2I region alone exhibited a strong proliferative response but only a relatively weak specific cytotoxic response. A cytotoxic response to H-2K/D-coded antigens, however, could be augmented by a concommitant anti-I region response. Cantor and Boyse (1975a, 1975b, 1976, 1977) have recently strengthened this hypothesis by the use of serological techniques which can, apparently, distinguish two distinct classes of T cells according to the expression of two sets of antigenic determinants (Ly-1 and Ly-2,3) on the cell surface. They have characterised a class of Ly-1^-, Ly-$2,3^+$ cytotoxic T cells, which respond to H-2K/D-coded antigens, and a class of Ly-1^+, Ly-$2,3^-$ non-cytotoxic T cells which respond to H-2I-coded determinants. From these observations, and the fact that a certain degree of synergistic activity can be demonstrated between Ly-1^+ and Ly-$2,3^+$ cells (Cantor and Boyse, 1975b), they have classified Ly-1^+ cells as helper T cells (T_H) and Ly-$2,3^+$ cells as cytotoxic T cells (T_C) as defined in the Bach T_H/T_C interaction model.

As it is defined this model rests firstly on the assumption that stimulation through differences at H-2I lead to responses of Ly-1^+ T_H cells, but *not* to Ly-$2,3^+$ T_C responses to any great extent. The evidence for this assumption is that T cells stimulated in MLC through H-2I express little cytotoxic activity for a variety of target cells of the appropriate H-2 genotype (Alter *et al.*, 1973; Schendel and Bach, 1974). Their explanation for this phenomenon was that cytotoxic T cells were not present in the culture.

Klein (1978) has severely criticised this model in expressing the view that no evidence yet exists either for the generation of a distinct class of helper T cells in MLC responses, or for their absolute requirement in these responses. He suggests that, contrary to other claims, stimulation in MLC through H-2I generates a strong cytotoxic response, as does stimulation through H-2K/D alone. Hence, there is no evidence for the existence of two *functionally* distinct classes of T cells (T_C and T_H) stimulated by H-2K/D and H-2I respectively.

We believe that the T_H/T_C interaction model as proposed by Bach and colleagues (Bach *et al.*, 1976, 1977) does not provide a tenable explanation for the observed phenomena. The fact that anti-I-region responses appear to involve only weak cytotoxic responses is quite likely due to an inadequate lysis assay rather than an absence of cytotoxic cells *per se*. Wagner *et al.*, (1975) showed that the type of target cell chosen for the cytotoxic assay had a large effect on the amount of anti-I region killing observed. Whereas concanavalin A- or LPS-induced blast targets allowed the detection of a significant level of anti-I killing, PHA-induced blasts did not. Davidson (1977) reported a high level of killing in MLC cultures between spleen cells from A.TH and A.TL mice, which differ only at the H-2I region and are otherwise congenic. This cytotoxic activity was detected using macrophage targets of stimulator type.

Another prediction of the 'Bach' model is that any cell, dead or alive, which carries on its surface I-region-coded

antigens (LD determinants) must be capable of stimulating in MLC. Hence the seemingly diverse range of metabolic inhibitors capable of destroying the stimulating capacity of lymphoid cells must do so by destroying or disrupting the I-region coded antigens, in most cases without affecting the K/D-region coded SD antigens. UV-irradiated spleen cells have been shown to be incapable of stimulating an allogeneic T_C response. Such cells, however, have serologically-detectable unaltered levels of SD antigens (Lindahl-Kiessling and Säfwenberg, 1972; Lafferty *et al.*, 1974) and are capable of stimulating full and specific cytotoxic responses in the presence of a third-party, allogeneic cell population (Lafferty *et al.*, 1974; Sopori *et al.*, 1977). This has been verified for allogeneic responses to heat-killed spleen cells (Schendel and Bach, 1974). These proposed different effects on SD- and I-region-coded (Ia?) antigens is not compatible with present indications that the two share a basically similar structure, suggesting that they are related, both evolutionarily and in how they are synthesized (Hess, 1976).

Another observation incompatible with this model is that in many cases, using congenic mouse strains, it has been shown that differences at either H-2K or H-2D alone are sufficient to stimulate significant T_C responses (Schendel *et al.*, 1973; Nabholz *et al.*, 1974; Forman and Klein, 1975; Melief *et al.*, 1975; Schendel and Bach, 1975; Davidson, 1977). Cantor and Boyse (1975b) found that Ly-1^+ T cells, proposed to be T_H cells, were not required for the activation of Ly-$2,3^+$ T_C responses under optimal culture conditions.

Bach *et al.*, (1977) have countered these observations by suggesting that H-2K/D-coded SD antigens may also have the capacity to activate helper T cells. If this were true it is hard to imagine why UV-irradiated or heat-killed cells, carrying a normal complement of SD antigens, have such a poor stimulating capacity.

1.6 THEORY OF ALLOGENEIC STIMULATION

Lafferty and co-workers have utilized the experimental system described by Nisbet and Simonsen (1967) to study the GVH response in the chick embryo, a system convenient for the study of the interaction of adult immunocompetent cells with a foreign but non-immunocompetent host (Lafferty *et al.*, 1972; Walker *et al.*, 1972, 1973). Based on the results of these studies a general theory of allogeneic reactivity has been proposed (Lafferty *et al.*, 1972; Lafferty and Cunningham, 1975; Lafferty and Talmage, 1976). This theory attempts to provide a rational explanation of both vertebrate and invertebrate alloreactivity and to link them via a basic underlying principle of immune reactivity.

When a GVH reaction is induced in the chick embryo with adult allogeneic cells a number of properties of the ensuing reactions can be observed. The most outstanding of these properties is that the reaction generally involves the proliferation of host rather than donor blood cell elements (Nisbet and Simonsen, 1967; Lafferty *et al.*, 1972). This indicates that these GVH reactions do not primarily involve the response of adult immunocompetent

cells to foreign host antigens. Rather, the adult spleen cells stimulate a response in the non-immunocompetent host-derived blood elements. This is the first principle upon which the theory of allogeneic reactivity was built. The capacity to stimulate in GVH reactions, but *not* the capacity to respond, is a property of mature (immunocompetent?) blood cells. More specifically, the theory postulates that allogeneic interactions are artifactual expressions of a general class of cell interactions in which cells of the lymphocyte/macrophage class control the activation of both nonimmunocompetent leucocyte precursors (nonspecific inflammatory reactions) and specific receptor-bearing immunocytes (adaptive immunity).

A second postulate of the theory is that nonspecific inflammatory reactions appeared early in metazoan evolution and that this primitive self-, not self-discrimination, based on a self-marker mechanism, has been further elaborated in the vertebrate animals to form the adaptive immune response. A number of invertebrates have the capacity to discriminate between self and not-self components with a considerable degree of specificity (Crichton and Lafferty, 1975). However, unlike vertebrates, the lower invertebrates show no evidence of an adaptive immune system, and in general, these animals show no immunological memory.

Among the colonial ascidians, tissue compatibility is controlled by a single genetic locus which has multiple alleles (A, B, C, ...). Provided animals share a single allele their tissues are compatible. Fully allogeneic colonies are incompatible and show a violent rejection response once their

blood systems have fused (Oka, 1970; Tanaka and Watanabe, 1973; Mukai and Watanabe, 1974; Tanaka, 1975). Following the mixing of haemolymph from incompatible colonies, haemolymph cells begin to aggregate and clump together. This is followed by a constriction of vessels in the region and final necrosis of the area (Tanaka, 1975). Tanaka described this phenomenon as a type of inflammatory reaction. An important observation with respect to ascidian compatibility reactions is that they appear to be mediated by haemolymph (blood?) cell interactions that result in a primitive inflammatory response (Oka and Watanabe, 1957; Mukai, 1967; Tanaka, 1975). Fusion characteristics of an animal of BC genotype can be modified by what amounts to a blood transfusion from a compatible animal AB. While the BC animal would normally be compatible with an animal of CD genotype, following the initial fusion with AB, the BC animal now containing AB haemolymph elements is no longer compatible with CD (Mukai, 1967). Clearly some blood element is playing a role of overriding importance in ascidian incompatibility reactions. These characteristics are very similar to the characteristics of the interaction between adult blood cell elements of the chicken and immature blood elements of the allogeneic chick embryo (Lafferty *et al.*, 1972; Walker *et al.*, 1972).

A further postulate of the theory, directly relevant to this study, is that vertebrate T cells may be activated through a similar mechanism involving a tissue compatibility locus (the MHC) like that described above for the colonial ascidians. Antigen specific T cells are activated when they interact with blood cells carrying a different or altered self-marker.

Activation does not occur when T cells interact with cells of non-haematogenous origin.

This hypothesis makes several predictions with respect to T cell responses, many of which have already been borne out. Activation of T cells can only be stimulated through a specialized stimulator cell, so that cells of non-haematogenous origin should not stimulate in MLC. Since activation of cells through the self-marker appears to involve an inductive signal the activation of T cells will depend on the metabolic status of the stimulating cells. Killed cells should not be able to stimulate T cell responses.

1.7 AIMS AND OUTLINES OF THESIS

The studies described in this thesis were undertaken in an attempt to define and characterise the requirements for the activation of cytotoxic T cells to foreign histocompatibility antigens *in vitro*. Studies of this type are often plagued by problems attributable to the complex experimental systems required for the generation of T cell responses in culture. In most studies a mixed population of spleen cells are cultured in the presence of foetal calf serum and 2-mercaptoethanol. Systems such as these are inherently complex and it is impossible to draw firm conclusions with respect to cellular and soluble requirements of T cell activation. We have attempted to reduce the complexity of this system to allow a greater definition of its component parts.

The first approach has been to use long-term cultured neoplastic cell lines as stimulator cells rather than mixed

lymphoid cell populations such as spleen or peripheral blood cells. This step defines the stimulating population as a homogeneous cell population rather than a complex and possibly interacting cell mixture. A second approach has been to use responding T cells from the lymph node; a secondary lymphoid organ, rather than the spleen, a primary haemopoietic organ. This step has been taken on the basis that lymph nodes are at least as rich in T cells as spleen but contain a less heterogeneous population of cells. This approach has been taken one step further in some experiments by the purification for T lymphocytes by nylon wool filtration.

By the combination of these two approaches it has been possible to reduce the *in vitro* response to allogeneic antigens largely to a two cell system - the homogeneous population of stimulating tumour cells and responding purified T cells. This system, in a number of experiments, has formed the basis of our studies.

The studies described in this thesis support the hypothesis that cytotoxic T cell responses, like the more complex GVH-type reactions described above, involve the interaction of blood cells rather than simply the recognition of foreign antigen by cytotoxic T precursor cells. Chapters 4 and 5 provide evidence for a two signal mechanism of activation. Signal 1 is provided by antigen while a second, inductive signal is normally provided by the stimulator cells but can also be prepared in soluble form. Both signals are required for T cell activation. Chapter 6 describes studies indicating that at least two separable activities, present in soluble preparations, play a

role in the generation of cytotoxic T cell responses. The suggestion is made that one activity is required for the induction of mature T_C effector cells while another is required to support their subsequent proliferation. The studies described in Chapter 7 provide direct evidence against the hypothesis that a distinct class of helper T cells are stimulated by I-region determinants in MLC.

In the final chapter these findings are discussed first with respect to their physiological relevance *in vivo* and second in the more general framework of allogeneic interactions described in Section 1.4. The precise mechanisms involved in these interactions are as yet unknown although it seems certain that MHC-defined products are involved in the recognition phase.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS

Mice of the DBA/2, BALB/c, C57Bl/6, A.TL, A.TH, SJL, C3H and CBA/H strains were used at 8-12 weeks of age. All mice were fed and watered *ad libitum*. The standard diet was Mecon rat and mouse cubes (Fidelity Feeds, Murrumburrah, N.S.W.).

Rats of the Black Hooded (BH), Lewis (Le) and DA strains were used at 11-14 weeks of age. These animals were maintained as described above.

Outbred guinea pigs were used at 12-20 weeks of age and were fed and watered *ad libitum*. The standard diet was Mecon rabbit and guinea pig cubes (Fidelity Feeds) supplemented with assorted greens and vegetables.

Randomly bred Merino Sheep, goats and Friesian cattle were maintained in the Animal Breeding Establishment of the John Curtin School of Medical Research as were all other animals used in these studies.

2.2 CULTURE MEDIA

Cell populations were generally first prepared in Hank's Balanced Salts Solution (BSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml neomycin (PSN). This medium will henceforth be referred to as Hank's BSS.

Foetal calf serum (Commonwealth Serum Laboratories, FCS) was heat inactivated by incubation at 56°C for 30 minutes.

Dulbecco's Modified Eagle's Medium (Gibco, H-16) was supplemented with 10% heat inactivated FCS (HIFCS) v/v and

PSN. This culture medium will henceforth be referred to as H-16 culture medium.

All lymphocyte cell cultures were carried out in Eagle's Minimal Essential Medium (Gibco, F-15) containing 10% HIFCS, 10^{-4}M 2-mercaptoethanol (SIGMA, M-6250) and PSN. This culture medium will henceforth be referred to as F-15 culture medium.

2.3 CELL CULTURE TECHNIQUES

All leucocyte cultures were carried out in a humidified atmosphere of 7% O_2, 10% CO_2 and 83% N_2 at 37°C.

Cell preparations were counted using a Trypan Blue exclusion test. 0.1 ml of the cell suspension was mixed with 0.1 ml of 0.5% Trypan Blue in Phosphate Buffered Saline (PBS) and an aliquot counted in a haemocytometer for total cell count and percent viable cells, excluding erythrocytes. In all cases at least 100 cells were counted.

2.4 MAINTENANCE OF TUMOUR CELL LINES

a) The DBA/2 mastocytoma cell line, P815-X2, the C57B1/6 lymphoma, EL-4 and the C58 lymphoma R1 were maintained *in vitro* in 25 cm^2 Falcon Tissue Flasks (Falcon, 3013) containing 5 ml Dulbecco's Modified Eagle's Medium (Gibco, H-16) supplemented with 10% v/v HIFCS.

Subculturing of the cell lines was performed twice weekly. Duplicate flasks were set up at 10^4 viable cells/ml and incubated under standard conditions (Section 2.3). When large number of P815 tumour cells were required for use as targets in cytoxicity assays or as stimulator

cells, they were subcultured into 30 ml of H-16 culture medium in 75 cm^2 flasks (Falcon, 3024), at an initial concentration of 10^4 cells/ml, and cultured under standard conditions for 3 days. On the day before use an equal volume of fresh culture medium was added to each flask.

b) The DBA/2 mammary carcinoma, CaD2, was obtained from Jackson Laboratories and maintained in DBA/2 mice as a solid tumour requiring passage by subcutaneous inoculation of 1 mm fragments every 2 weeks. An *in vitro* cell line growing as a monolayer with a doubling time of approximately 12 hr was obtained by incubating small fragments of the tumour at 37°C with 0.1% trypsin for 30 minutes in Puck's saline A (Gibco) and then washing and culturing the cells in H-16 culture medium. A single cell suspension was obtained for passage, for stimulating lymphocytes in culture, and for labelling for cytotoxic assays by incubating the monolayers with 0.1% trypsin for 20 minutes. The cells were then washed twice with Hank's BSS and counted. The CaD2 cells used in these experiments had been grown *in vitro* by weekly transfer for more than 3 months. All tumour cell lines were passaged through syngeneic mice at least twice yearly.

2.5 PREPARATION OF NORMAL CELL POPULATIONS

Mouse peritoneal cells were obtained by washing the peritoneal cavity of normal mice with 5 ml Hank's BSS containing heparin at 20 units/ml.

Mouse, rat and guinea pig spleen and lymph node suspensions were prepared by removing the organs aseptically, trimming them of excess fat, and pressing through a fine stainless steel seive into fresh Hank's BSS. The resultant cell suspension was transferred to a graduated centrifuge tube and allowed to settle on ice for 5 minutes to remove debris and cell clumps. The supernatant, a single cell suspension, was transferred to a fresh centrifuge tube and spun at 1200g for 5 minutes. The cell pellet was resuspended in 10 ml F-15 culture medium.

Lymph node cell suspensions were made from mouse and rat mesenteric, axillary and inguinal nodes or guinea pig cervical nodes.

Bovine spleen cells were prepared by grinding small fragments of spleen in a glass homogenizer.

Erythrocytes were removed from guinea pig and bovine spleen cell suspensions by centrifugation and resuspending the pellets in 0.85% ammonium chloride for 10 minutes at 37°C. The cell suspensions were then washed with two changes of Hank's BSS and finally resuspended in fresh F-15 culture medium.

Human peripheral blood buffy coat was obtained from the Canberra Hospital Blood Bank and treated with 0.85% ammonium chloride to lyse erythrocytes as described above.

Sheep and goat peripheral blood was taken from the jugular vein and separated on Ficoll/Isopaque using a modification of the method described by Davidson and Parish (1975). Five ml of the blood suspension was layered gently onto 4 ml of separating medium which had been pre-warmed to 20°C and which

consisted of 12 parts of 14% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) dissolved in distilled water, and 5 parts of 32.8% (w/v) sodium metrizoate (Isopaque, Nyegaard and Co., Oslo, Norway). The medium was sterilized by millipore filtration. Separation was carried out in conical glass centrifuge tubes at 2500g for 30 minutes. Lymphocytes remained at the Isopaque/Ficoll interface, while erythrocytes pelleted. The lymphocyte layer was collected, washed 3 times (1200g, 5 minutes) in 10 ml of Hank's BSS, and resuspended in culture medium. An aliquot of the suspension was counted by Trypan Blue exclusion (Section 2.3), and the remainder diluted to the required concentration with F-15 culture medium.

2.6 IRRADIATION OF STIMULATOR CELLS

Tumour cells were γ-irradiated with 5,000 R and normal leucocytes with 850 R from a ^{60}Co source. Cells were irradiated with ultraviolet radiation (UV) in a suspension 1 mm deep for 4 minutes with a 30-watt germicidal lamp at an intensity of 960 $\mu W/cm^2$ in the 230 to 270 nm range.

2.7 GENERATION AND ASSAY OF MIXED LYMPHOCYTE RESPONSES (MLR)

Mixed cell cultures were prepared by mixing equal volumes of stimulating and responding cell suspensions at the required densities in F-15 culture medium (Section 2.2). 0.2 ml aliquots were added to three replicate wells of Linbro 16 mm microtitre plates (Linbro, IS-FB-96TC). The culture trays were placed at 37°C under standard culture conditions (Section 2.3). After the required periods, 5 μCi of ^{3}H-thymidine (Methyl ^{3}H-thymidine, aqueous solution, The Radiochemical Centre,

Amersham, U.K.) was added to each well. Trays were then returned to gassed boxes at 37°C for 5 hours. After this time, the contents of individual wells were harvested onto glass fibre filter strips (Reeve Angel, 934-AH) and washed with 0.9% w/v NaCl in distilled water, using a Multiple Automated Sample Harvester (MASH-II, Microbiological Associates, U.S.A.). Filter strips were dried, then the discs bearing the samples were cut out and placed in scintillation vials containing 0.5% w/v 2,5-diphenyloxazole (PPO, KOCH-Light Laboratories Ltd. or Packard Instrument Co. Inc.) in toluene. Samples were counted in a Packard Liquid Scintillation Spectrometer for 1-10 minutes each. ^{3}H-thymidine uptake was expressed as mean counts per minute in triplicate samples ± standard error.

2.8 GENERATION AND ASSAY OF CYTOTOXIC CELLS

γ-irradiated or UV-irradiated tumour cells were cultured with responding lymph node or peripheral blood cells (10^6) in 1 ml F-15 culture medium in Multiwell tissue culture plates (Falcon, 3008) and incubated under standard culture conditions (Section 2.3).

Target cells were labelled with $Na^{51}CrO_4$ (Institut National des Radioelements, Belgium, Cat. No., CR-51-S1, 100 µCi/ml) at a cell density of $5x10^6$/ml in H-16 culture medium containing 5% HIFCS. Tubes were gassed with 10% CO_2, 7% O_2 and 83% N_2, sealed and incubated at 37°C for 60 minutes. Cells were then washed three times in 10 ml of H-16 containing 5% HIFCS, centrifuging at 1000g for 5 minutes, and finally resuspended in

F-15 culture medium (without 2-ME) at a concentration of 10^6/ml.

Cytotoxic cell populations were suspended in F-15 containing 10% HIFCS at appropriate dilutions and 0.1 ml of each dilution was added to quadruplicate wells of flat-bottomed 96 well culture trays (Linbro, IS-FB-96TC). To each well was added 0.1 ml of the target cell suspension. Spontaneous ^{51}Cr-release was determined by adding 0.1 ml of F-15 containing 10% HIFCS to 4 wells containing 0.1 ml of target cell suspension, and total releasable ^{51}Cr was determined by adding 0.1 ml target cell suspension to plastic centrifuge tubes containing 0.9 ml distilled water. All mixtures were incubated at 37°C for 4 hours under standard culture conditions (Section 2.3). Aliquots of 0.1 ml of the supernatants were taken and counted in a Packard Gamma Scintillation Spectrometer for one minute each. Water lysis tubes were vortexed and centrifuged at 3,000g for 10 minutes. 0.5 ml aliquots were removed and counted as described above.

Cytotoxic activity was generally expressed as cytotoxic units per culture (CU/culture), where 1 CU is that activity required to lyse 1 target cell under the conditions of the assay. The derivation of this unit of activity and our reasons for using it are discussed in Chapter 3.

The formula used to calculate CU/culture is:

$$\text{CU/culture} = \frac{-Y_o \ln(1-y)}{f}$$

where Y_o = the number of target cells in the assay,

f = the fraction of the original culture assayed,

and y = the fraction of targets specifically lysed during the 4 hour assay

$$= \frac{\text{(Test lysis - Control lysis)}}{\text{(Water lysis - Control lysis)}}$$

This unit of cytotoxic activity is directly proportional to the number of cytotoxic cells being assayed and cultures demonstrating a cytotoxic activity of 10^5 CU/culture caused approximately 33% specific lysis of target cells at an effector to target ratio of 1:1.

2.9 PREPARATION OF CONCANAVALIN A-ACTIVATED CELL SUPERNATANT (CS)

CS was prepared using a modification of a method described by Pick and Kotkes (1977). Spleen cells or peripheral blood leucocytes (10×10^6/ml in 30 ml aliquots) were cultured in serum-free F-15 medium containing 10^{-4}M 2-ME and concanavalin A (Con A, Gibco C-2010) at a final concentration of 5 µg/ml for cells of mouse and rat spleen and 10 µg/ml for cells of other species. All CS preparation was carried out in the absence of foetal calf serum. Cultures were set up in 75 cm^2 plastic culture flasks (Falcon, 3024), and maintained at 37°C in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2 for 2 hours. At this time most cells had adhered to the plastic substrate and the medium was discarded. The cell monolayer was then washed gently three times with warm Hank's BSS and replenished with 30 ml fresh serum-free F-15 culture medium containing 10^{-4}M 2-ME. The flasks were incubated at 37°C for a further 17-20 hours in a humidified atmosphere of 7% O_2, 10% CO_2 and 83% N_2.

The supernatant was harvested, centrifuged to remove any cells and concentrated at least 10-fold on an Amicon PM-10 membrane. The concentrated CS preparation was sterilized by filtration and stored at -20°C. The preparation was added to cultures at a concentration of 10% by volume.

2.10 LIMITING DILUTION ASSAY

Responding lymph node cells were prepared in F-15 culture medium (Section 2.2) at appropriate cell densities. Stimulating cells were γ-irradiated and adjusted to a concentration of $10^{5.5}$/ml for tumour cells and $10^{6.5}$/ml for spleen cells in the same culture medium containing 20% CS (v/v). 0.1 ml each of the responding and stimulating cell suspensions were added to individual wells of flat-bottomed 96 well culture trays (Linbro, IS-FB-96TC) and incubated for four days at 37°C under standard conditions (Section 2.3). On day 4, 0.1 ml of the supernatant was removed from each well without disturbing the cells and replaced with 0.1 ml fresh F-15 culture medium (Section 2.2) containing 20% CS (v/v). The cultures were incubated for a further 3 days.

On day 7 individual wells were assayed for cytotoxic activity against ^{51}Cr-labelled P815 or EL-4 target cells. 0.1 ml of the supernatant was removed from each well and replaced with 0.1 ml F-15 containing 10% HIFCS and 10^5 ^{51}Cr-labelled target cells (Section 2.8). The cultures were incubated for 5 hours as described in Section 2.3. Wells causing a specific ^{51}Cr-release greater than 2 standard deviations above control cultures originally containing responding cells alone were scored as positive.

For some experiments individual wells were examined visually with an inverted light microscope for the presence of activated blast cells. Those wells containing more than 50 blasts were scored as positive.

The frequency of reactive clones was determined mathematically from the distribution of non-reactive wells as described by Groves, Lever and Makinodan (1970). The derivation of this technique which is based on Poisson statistics is described in Appendix I.

2.11 TREATMENT WITH ANTI-THY 1.2 SERUM AND COMPLEMENT

An aliquot of the cell cultures to be tested were incubated with an AKR anti-C3H ascites (Kirov, 1974) or a control AKR ascites for 20 minutes at 37°C. The cells were then washed and guinea pig complement absorbed with responder strain spleen cells was added (final concentration 1:3). The cells were incubated a further 20 minutes at 37°C, washed and the cells counted using the Trypan Blue exclusion test (Section 2.3).

CHAPTER THREE

QUANTITATIVE ASSAY OF CYTOTOXIC ACTIVITY ; DEFINITION OF A CYTOTOXIC UNIT

3.1 INTRODUCTION

The study of cytotoxic cells requires a reliable and reproducible assay for the quantification of effector cell function. This applies equally for studies carried out *in vitro* or *in vivo*. Whether studies are concerned with the very early events of cytotoxic cell induction or with the later event of the cytolytic process itself, they rely heavily on the ability to measure the efficiency with which the cytotoxic cells lyse specific target cells. For these reasons the development of the chromium-release assay over the last decade has greatly facilitated our investigations of this aspect of cellular immunology. Brunner *et al* (1968) found that when ^{51}Cr-labelled target cells (mouse P815 mastocytoma) were mixed with a population of lymphocytes sensitized *in vivo* to P815, the observed release of ^{51}Cr into the medium was directly related to target cell inactivation as measured using a growth inhibition assay. This assay, the ^{51}Cr-release assay as described by Brunner and co-workers in 1968, still forms the basis for the estimation of cytotoxic cell activity, which is generally expressed in terms of percent specific lysis of the target cells.

The major drawback of this assay system is that, although an accurate estimate of the number of targets killed can be made, there is no simple relationship between percent specific lysis and number of effector cells present in a preparation. One approach to overcome this problem has been to express cytotoxic activity in terms of lytic units which are directly proportional to the number of effector cells present in the

assay (Shortman *et al.*, 1972; Engers and MacDonald, 1976). In this type of assay the effector populations to be tested are titrated in a standard cytotoxic assay to determine the dilution required to cause an arbitrarily defined level of target cell lysis. One lytic unit is defined as that amount of cytotoxic activity required to cause a given degree of target cell lysis under fixed assay conditions. The advantage of this system is that cytotoxic activity, expressed in terms of lytic units, is now directly proportional to the number of effector cells tested. This allows a true comparison between the cytotoxic activities of different cell preparations, a comparison which cannot be made using percent specific ^{51}Cr-release as the basic unit of measurement.

We have also adopted a unit of cytolytic activity. This cytotoxic unit (CU) is defined as the activity required for the lysis of one target cell under standard assay conditions. Using this unit, cytolytic activity can be derived mathematically from the specific lysis of target cells obtained in single point assays. The advantage of this unit is that it elliminates the need for complete dose response curves for each estimate. A detailed description of the theoretical derivation of this unit will be described in Section 3.6.

3.2 DEFINITION OF THE CYTOTOXIC UNIT

It has been shown empirically by Henney (1971) that the interaction between effector and target cells can be closely fitted to an expression of the form

$$f = 1 - e^{-\alpha.N} \qquad [1]$$

where f = the observed specific fractional ^{51}Cr-release,

N = the number of sensitized lymphocytes in the assay,

and α = a constant.

Based on theoretical considerations, Miller and Dunkley (1974) have proposed that these interactions can be described by essentially the same exponential function. Thus,

$$f = 1 - e^{-n.\delta.t} \qquad [2]$$

where f = the fraction of target cells destroyed,

n = the number of effector cells,

t = the duration of the assay,

and δ = a constant interaction probability.

If it is assumed that these interactions behave as a first order reaction, it can be shown (Section 3.6) that this equation can be expressed in the form

$$y = 1 - e^{-C.N.\mu.t/Y_o} \qquad [3]$$

where y = the specific fractional lysis,

C = the number of effector cells present in the assay,

N = the number of targets contacted by one killer in unit time,

μ = the probability that contact with a live target causes lysis,

t = the duration of the assay,

and Y_o = the number of live, labelled targets originally available for lysis.

One cytolytic unit (CU) is the cytotoxic activity required to lyse one target cell under the conditions of the assay. Hence, the number of cytotoxic units per assay equals the number of targets killed per unit time at time zero (t_o), times the duration of the assay (t).

$$\text{i.e.} \quad CU = C.N.\mu.t \qquad [4]$$

CU is thus, directly proportional to the rate of target cell lysis at t_o, which in itself is an expression of the capacity of the sensitized population to lyse target cells at the beginning of the assay when the number of target cells is not limiting. Also, CU is linearly related to the number of effector cells (C). Substituting in equation [3], we get

$$y = 1 - e^{-(CU)/Y_o} \qquad [5]$$

From this we see that

$$CU = -Y_o \ln(1-y) \qquad [6]$$

where CU = the number of cytotoxic units in the assay,

Y_o = the number of viable, labelled target cells originally available for lysis,

and y = the fraction of target cells destroyed as determined by specific release of ^{51}Cr.

CU is linearly related to the number of effector cells (C) present in the assay (equation 4) under constant culture conditions, and can be derived directly from a single estimate of fractional lysis (y).

3.3 METHODS

3.3.1 *Generation of cytotoxic T cells.*

This was carried out as described in Section 2.8. γ-irradiated P815 ($10^{5.2}$/ml) and C57Bl/6 lymph node cells (10^{6}/ml) were mixed in triplicate one ml cultures in F-15 culture medium and incubated under standard conditions (Section 2.3) for five days.

Effector populations were tested for cytotoxic activity against ^{51}Cr-labelled P815 also as described in Section 2.8.

3.3.2 *Growth inhibition assay.*

0.1 ml volumes of the cell suspensions to be tested were added to individual wells of a flat-bottomed 16 mm culture tray (Linbro, IS-FB-96-TC) containing 10^{5} unlabelled P815 in 0.1 ml F-15 culture medium (without 2-ME). The trays were incubated for 4 hours under standard conditions (section 2.3) and were then assayed for target cell inactivation. Cells were harvested from each well and 0.1 ml volumes of the cell suspensions were diluted in 5 ml of warm H-16 culture medium containing 0.3% agar (DIFCO, Bacto-agar). Two ml volumes of this cell suspension were then transferred to plastic 35 mm culture trays (Linbro, FB-6-TC), allowed to cool for 15 minutes, then incubated under standard conditons (Section 2.3) for 7 days. On day 7 colonies were counted using a binocular dissecting microscope (20x). Target cell inactivation was estimated from the number of colonies from test wells as compared to the number of colonies from control wells containing P815 alone.

3.4 EMPIRICAL ANALYSIS

This section describes experiments designed to test the validity of the mathematical model described above as applied to the lysis of target cells in a ^{51}Cr-release assay.

3.4.1 *Relationship between target cell death and ^{51}Cr-release from target cells.*

The first experiment was designed to test whether in our system the fractional lysis of target cells as determined using a ^{51}Cr-release assay agreed with that determined using a growth inhibition assay. C57Bl/6 anti-P815 cytotoxic cells were generated using the standard *in vitro* technique (Section 3.3.1). Serial 2-fold dilutions of a suspension from this culture were made and assayed for cytotoxic activity in two ways, using the ^{51}Cr-release (Section 3.3.1) or growth inhibition (Section 3.3.2) assays. Table 3.1 describes the results of this experiment. Target cell death as assayed using a colony inhibition assay agreed very closely with the ^{51}Cr-release assay. Hence, it can be concluded that the latter gives an accurate estimate of target cell death through cell-mediated cytolytic activity.

3.4.2 *Experimental validation of the model.*

The following experiment was designed to test whether the theoretical model proposed above fits the experimentally observed lysis of target cells. According to our model the number of cytotoxic units (CU) observed in an assay of constant time (t) is a linear function of the number of effector cells in the killer population (C) (equation 4). Another corollary

TABLE 3.1

Estimation of target cell death using
i) ^{51}Cr-release assay and
ii) an agar colony growth inhibition assay

		Growth inhibition	
Log_{10} dilution of effector cells	Specific ^{51}Cr-release	Colony number	% colony inhibition
0.0	100%	4	98%
0.3	75%	54	76%
0.6	51%	101	55%
0.9	29%	166	26%
Control	0%	224	0%

Cytotoxic cells were generated in 1 ml cultures containing $10^{5.2}$ γ-irradiated P815 and 10^{6} C57Bl/6 lymph node cells. After 5 days in culture effector cells were tested for cytotoxic activity against ^{51}Cr-labelled or unlabelled P815 in 0.2 ml volumes over a 4 hr incubation period. Lysis of ^{51}Cr-labelled targets was estimated from ^{51}Cr-release into the supernatant. Lysis of unlabelled targets was estimated by measuring the cloning efficiency of remaining target cells in 0.3% agar in H-16 + 10% FCS.

Total releasable ^{51}Cr as estimated by water lysis was 6,800 cpm. Control wells containing 10^{5} ^{51}Cr-labelled targets in 0.2 ml culture medium gave a ^{51}Cr-release of 869 cpm.

of the above model is that the two functions describing

(A) the number of cytotoxic units (CU), and

(B) the number of target cells lysed (Y_c),

with respect to effector cell number, should have the same slope at the origin, that is, when effector cell number (C) tends to zero (Section 3.6). These functions are described by the following equations.

$$(A) \quad CU = C.N.\mu.t \qquad [4]$$

$$(B) \quad Y_c = Y_o - Y_o.e^{-C.N.\mu.t/Y_o} \qquad [7]$$

The derivatives with respect to C of both these equations have the value N.μ.t when C→0 (Section 3.6). To test this prediction a population of C57Bl/6 anti-P815 cytotoxic cells were generated using the standard *in vitro* activation technique (Section 3.3.1). Figure 3.1 describes the data from this experiment in two different ways. The solid curve shows the number of target cells killed (Y_c) by different numbers of effector cells. This is estimated from ^{51}Cr-release according to the following equation.

$$Y_c = Y_o.y \qquad [8]$$

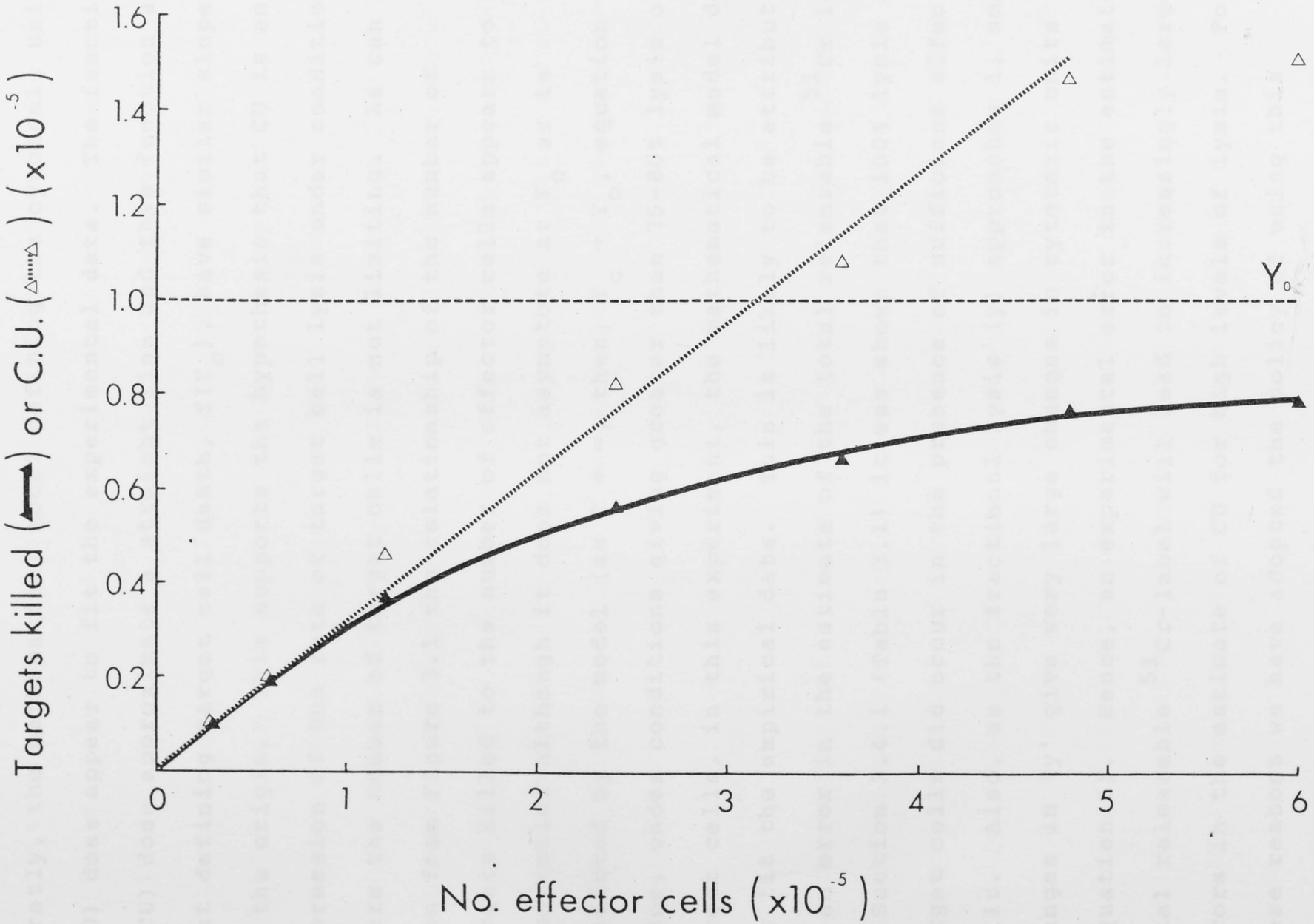
where Y_o = the number of target cells originally available for lysis

and y = fractional specific lysis, and is determined from the equation

$$y = \frac{\text{test release - control release}}{\text{full release - control release}} \qquad [9]$$

These estimations are described in Section 2.8. The broken line of Figure 3.1 shows the cytotoxic activity expressed in terms of

Figure 3.1 Dose response curves of the lytic activity of anti-P815 cytotoxic effector cells expressed in terms of a specific ^{51}Cr-release (▲——▲) and b) cytotoxic units (CU) per culture (Δ---Δ) using the formula $CU = -Y_o \frac{\ln(1-y)}{f}$ (see Section 3.6). Effector cells were generated in 1 ml cultures containing $10^{5.2}$ γ-irradiated P815 and 10^6 C57Bl/6 lymph node cells. Cytotoxic activity was estimated by mixing different dilutions of effector cells with 10^5 ^{51}Cr-labelled P815 target cells in 0.2 ml F-15 culture medium containing 10% FCS. Target cell lysis was estimated by measuring ^{51}Cr-release into the supernatant.


Targets killed (⟷) or C.U. (△⋯△) (x10⁻⁵)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Y₀
No. effector cells (x10⁻⁵)

cytotoxic units (CU) as defined by equation [6].

A number of conclusions can be drawn from the data. Firstly, the mathematical model defining the cytotoxic unit (CU) does appear to fit the experimental data. The function f(CU) does approximate a straight line and this function and that defining target cell death, $f(Y_c)$, have similar slopes at the origin. This supports the hypothesis that CU is an expression of the rate of target cell lysis under conditions where the number of target cells is not limiting. As can be seen from Figure 3.1 the relationship of the number of targets killed to the number of effector cells appears to be exponential although it does not asymptote at Y_o as is predicted by the model (as $C \rightarrow \infty$, then, $Y_c \rightarrow Y_o$, equation 7). Hence, under conditions giving greater than 75-80% lysis of target cells, in this experiment, the mathematical model does not fit the empirical data. This is likely to be attributable to an error in the estimate of the total releasable ^{51}Cr label. In Section 3.4.1 (Table 3.1) it was shown that 100% lysis of target cells did occur in the presence of sufficient effector cells. Also, as the fractional lysis (y) approaches 1, small changes in 'y' give very large changes in cytotoxic units (CU) (equation 6). Hence, an experimental error in the estimate of total releasable ^{51}Cr-label will lead to increasingly large errors in the estimate of CU for high levels of lysis. For these reasons we have adopted the policy of using this mathematical model only under conditions giving less than 80% specific lysis of target cells (i.e., where $y < 0.8$).

3.4.3 *Variability of the* in vitro *cytotoxic response.*

To test the variability of cytotoxic assays, 24 wells, each containing γ-irradiated P815 ($10^{5.2}$) and C57Bl/6 lymph node cells (10^{6}) in 1 ml F-15 culture medium, were set up as described in Section 2.8. These cultures were assayed as 8 replicate assays, each assay being performed in triplicate as described in Section 2.8. On day 5 the 8 replicate cultures were assayed for cytotoxicity to P815 and variation between assays was determined. Table 3.2 shows data from four such experiments. The standard deviation of replicate cultures was 0.06 to 0.07 Log_{10} cytotoxic units when the activity of cultures was in the range of 10^{5} CU/culture. This variation increased as cytotoxic activity was estimated from low levels of specific lysis (<5%).

3.5 CONCLUSIONS

The theoretically-defined cytotoxic unit (CU) greatly facilitates the quantitative analysis of cytotoxic cell cultures over a wide range of activities. The use of this unit allows the direct comparison of different cultures, which is impossible using specific ^{51}Cr-release at arbitrary effector to target ratios without extensive dose response analysis. Furthermore the sensitivity of this assay can be controlled simply by adjusting the fraction of the culture assayed (f).

This unit of cytotoxic activity has been used in all experiments in this thesis except where otherwise indicated.

TABLE 3.2

Variation of cytotoxic T cell responses in vitro

		Log_{10} C.U./culture†	
	%Lysis	Mean	Std. Deviation
Experiment 1	36%	5.17	0.06
Experiment 2	28%	5.04	0.07
Experiment 3	8.4%	4.46	0.18
Experiment 4	2.3%	3.77	0.19

† Each value is derived from a pool of eight individual cultures, each culture consisting of 3 wells. Cytotoxic activity was calculated using the formula $CU/culture = -\frac{YoLn(1-y)}{f}$ as described in Section 3.6. Cytotoxic assays were carried out as described in Section 2.8.

3.6 QUANTITATION OF KILLING ACTIVITY *IN VITRO:* A THEORETICAL ANALYSIS

The cytotoxic activity of a killer population is expressed in terms of the number of cytotoxic units (CU) per culture well. One cytotoxic unit is defined as that entity capable of lysing one target cell. All cytotoxic activities are expressed as a function of the number of cells in the original, unstimulated responding population in the MLC.

The following derivation of the cytotoxic unit rests on two basic concepts. It is assumed that the amount of ^{51}Cr released from a target cell is the same by all types of lysis: water, spontaneous, or cell-mediated. Also, it has been shown (Henney, 1971; Lafferty, *et al.*, 1974) that in regions of target cell excess, a plot of log (specific ^{51}Cr release) versus log (no. of killers present) was a straight line with slope approximating 1. This indicated that the lysis process was a 'one hit' phenomenon, requiring no interaction between cells in the killer population.

Our basic assumption is that the number of targets killed, and hence the amount of chromium released, in unit time is directly proportional to:

a) the no. of killers in the assay (C).

b) the no. of targets one killer can contact in unit time (N).

c) the probability that a killer/target contact involves a live target (P)

d) the probability that live contact causes lysis (μ).

If we let

Y_o = no. of targets available for lysis at the beginning of the assay (t_o).

Y_c = no. of targets specifically lysed by C killer cells in time t

and α = ^{51}Cr release from one lysed target cell, then

$\alpha.Y_o$ = total specifically releasable ^{51}Cr,

$\alpha.Y_c$ = ^{51}Cr released by Y_c target cells,

and $\frac{Y_o - Y_c}{Y_o}$ = prop. of live targets remaining in the assay

= P.

Now, assuming that no other factors are involved, then the no. of targets killed per unit time

$$= C.N.\mu.P$$

$$= C.N.\mu.\left(\frac{Y_o - Y_c}{Y_o}\right)$$

i.e. the rate of change of the no. of surviving targets

$$Y_o - Y_c = -C.N.\mu\left(\frac{Y_o - Y_c}{Y_o}\right)$$

i.e. $$\frac{d(Y_o - Y_c)}{dt} = -C.N.\mu\left(\frac{Y_o - Y_c}{Y_o}\right).$$

This is a differential equation of the form $f'(t) = a.f(t)$, which has as a general solution: $f(t) = k.e^{a.t} + k'$ (where k and k' are constants) by the chain rule of differentiation.

Hence, $Y_o - Y_c = k.e^{-C.N.\mu.t/Y_o} + k'$.

Now, as $t \rightarrow \infty$, $Y_c \rightarrow Y_o$ (all targets lysed)

$\therefore$ $(Y_o - Y_c) \rightarrow 0$ and $k.e^{-C.N.\mu.t/Y_o} \rightarrow 0$

$\therefore$ $k' = 0$

Also, when $t = 0$, $Y_c = 0$ (No targets lysed)

$\therefore$ $k = Y_o$

We now have an equation of the form

$$Y_o - Y_c = Y_o.e^{-C.N.\mu.t/Y_o}$$

or $$Y_c = Y_o - Y_o.e^{-C.N.\mu.t/Y_o} \qquad [10]$$

We define one cytotoxic unit as the capacity to kill one target under the conditions of the assay. Hence, the number of cytotoxic units (CU) per assay equals the number of targets killed per unit time at time zero (t_o), times the duration of the assay (t).

i.e. $$CU = C.N.\mu.t \qquad [4]$$

Therefore, $$Y_o - Y_c = Y_o.e^{-CU/Y_o}$$

Therefore, $$1 - \frac{Y_c}{Y_o} = e^{-CU/Y_o}$$

Now, if we assume that

$$\frac{Y_c}{Y_o} = \frac{\alpha.Y_c}{\alpha.Y_o}$$

$$= \frac{\text{specific } {}^{51}Cr \text{ release}}{\text{total specifically releasable } {}^{51}Cr}$$

and let $\frac{\alpha.Y_c}{\alpha.Y_o} = y$,

then, $1 - y = e^{-CU/Y_o}$

therefore,

$$\ln(1-y) = \frac{-CU}{Y_o}$$

therefore,

$$CU = -Y_o.\ln(1-y).$$

i.e. No. of cytotoxic units per C killer cells in time

$$t = -Y_o.\ln(1-y)$$

where $y = \frac{\alpha.Y_c}{\alpha.Y_o}$ at time t.

Therefore, no. of CU per MLC culture well

$$= \frac{-Y_o.\ln(1-y)}{f} \qquad [11]$$

where f = fraction of the MLC well assayed.

According to this mathematical model the number of cytotoxic units in an assay of constant time t is a linear function of the number of cells in the killer population (C).

$$CU = C.N.\mu.t$$ where N, μ and t are constants (equation 4).

i.e. $$\frac{d\ CU}{d\ C} = N.\mu.t$$

= No. CU per cell in the killer population.

Also, the number of targets killed in an assay of constant time t is an exponential function of the number of cells in the killer population (C).

$$Y_c = Y_o - Y_o.e^{-C.N.\mu.t/Y_o} \qquad \text{(equation 10)}$$

i.e. $$\frac{d\ Y_c}{d\ C} = N.\mu.t.e^{-C.N.\mu.t/Y_o}$$

Now, when C = 0

$$\frac{d\ CU}{d\ C} = N.\mu.t$$

and $$\frac{d\ Y_c}{d\ C} = N.\mu.t$$

i.e. the two functions have the same slope at the origin

i.e. when the number of cells in the killer population (C) tends to zero.

CHAPTER FOUR

CYTOTOXIC T CELL RESPONSES TO NORMAL AND ONCOGENIC CELLS

4.1 INTRODUCTION

When T cells are mixed in culture with leucocytes carrying foreign transplantation antigens some are activated and differentiate into cytotoxic cells reactive to the stimulating antigens (Häyry and Defendi, 1970). Early attempts to explain this phenomenon were based on the assumption that recognition of antigen *alone* induced the differentiation of lymphocyte clones (Medawar, 1963). This assumption is not supported by experimental observation. Only viable allogeneic leucocytes have the capacity to stimulate T cell responses *in vitro* (Section 1.4) or *in vivo* (Section 1.3).

In the light of this and other evidence Lafferty and co-workers (Lafferty, *et al.*, 1972) suggested that allogeneic interactions, resulting in lymphocyte activation, represent a special class of cell interaction in which the cell that provides the source of foreign antigen also provides an inductive signal for the responding cell. That is, a specialized stimulator cell is required for the induction of allogeneic lymphocytes *in vitro*. Stimulator cells are those cells which provide a source of the inductive signal as well as antigen. Experimentally, stimulator cells are cells of the lympho-reticular class (Greineder and Rosenthal, 1975; Hardy and Ling, 1969). More recently Lafferty and Cunningham (1975) developed a more general theoretical framework that relates alloreactivity to the normal process of antigenic induction in the immune system. This model of immune induction is based on three postulates:

1. Normal antigen induction is a two signal process requiring cellular interactions within the lymphoid system that are triggered by the entry of foreign antigen (Figure 4.1). Immune induction occurs when the responsive cell binds antigen through its surface receptor (signal 1) and simultaneously receives an inductive stimulus (signal 2) from the stimulator cell, which is also able to bind antigen to its surface.

2. Allogeneic interactions are an artefactual expression of this normal inductive process. Allogeneic interactions occur when foreign antigen (transplantation antigens) is present as an integral surface component of the stimulating cell; stimulator cells are cells with the capacity to produce and release the second signal (Figure 4.2).

3. The species specificity of allogeneic interactions merely reflects the species specificity of the second signal.

The studies described in this chapter were designed to examine the second postulate of the theory of allogeneic reactivity, described above, using cloned tumour cell lines to stimulate allogeneic T cells *in vitro*. According to this theory, tumour lines should fall into two classes, stimulatory (S^+) or non-stimulatory (S^-), depending on their tissue origin. Tumours of lympho-reticular origin would be expected to express the S^+ phenotype and tumours derived from tissue parenchymal cells would be expected to be S^-. Expression of the S^+ phenotype is dependent on the metabolic activity of the stimulating tumour and provision of an exogenous source of the second signal should facilitate T cell activation by S^- tumours.

Figure 4.1 Normal antigen induction occurs when the responsive T cell binds antigen (Ag) to its surface receptor (signal 1) and simultaneously receives an inductive stimulus (signal 2) from the stimulator cell (S^+) which also carries a receptor for antigen on its surface. The delivery of both signals results in T cell activation.

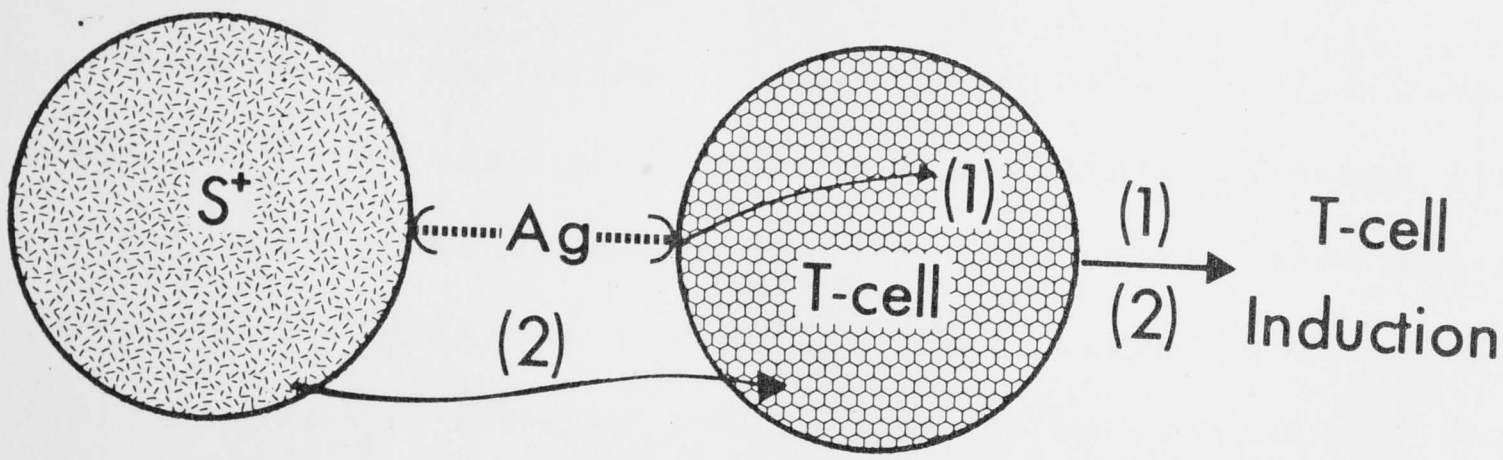
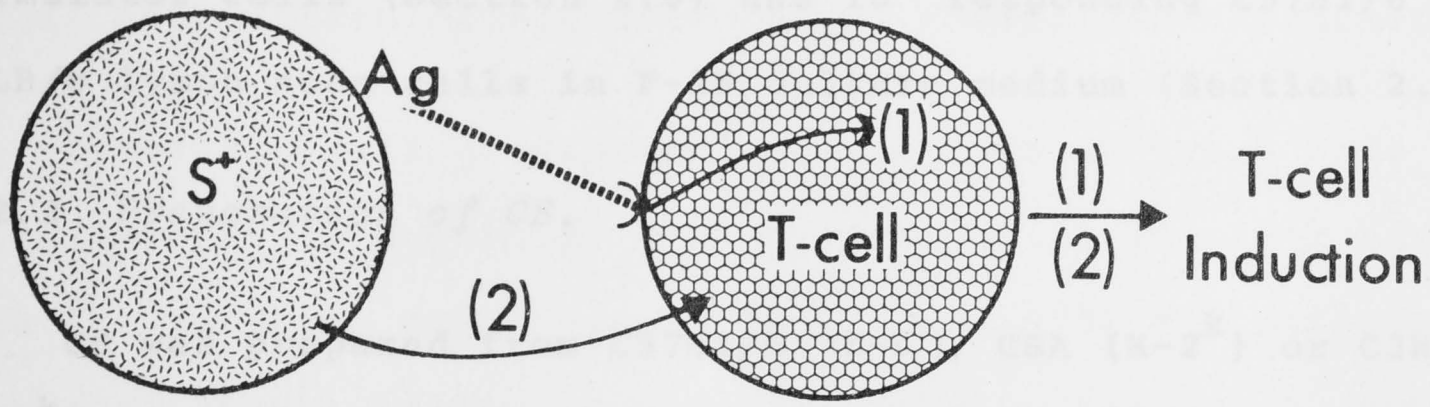
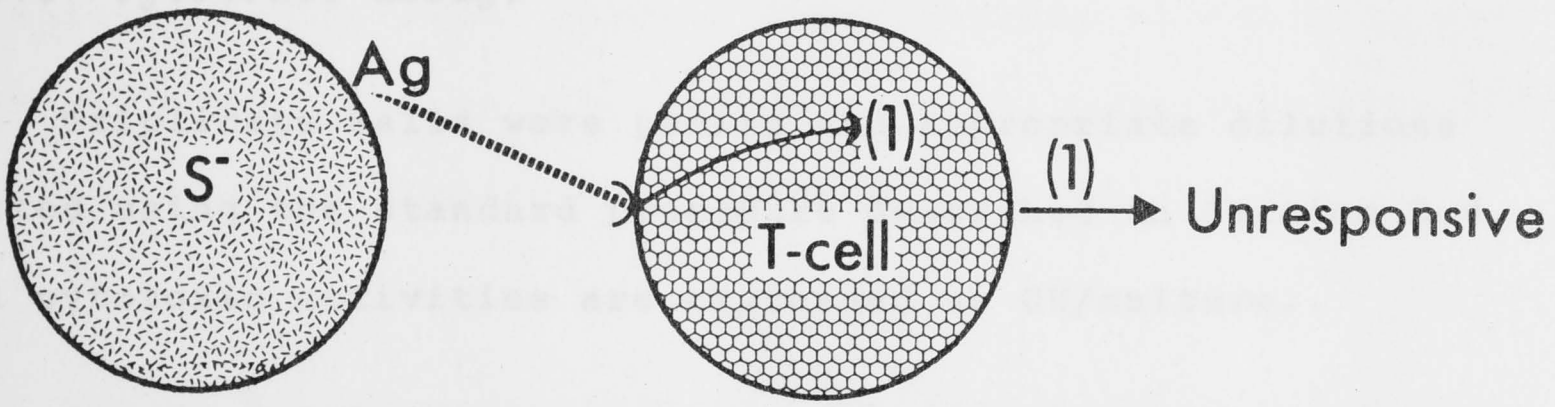

S+
Ag
(1)
T-cell
(2)
(1)
(2)
T-cell
Induction

Figure 4.2 Allogeneic T cell activation occurs when the T cell receptor binds antigen (signal 1) carried on the surface of the stimulator cell (S^+) and simultaneously receives signal 2 from the S^+ cell. S^- cells fail to stimulate because they lack the ability to produce and/or release the second signal required for T cell activation.


Ag
S+
(1)
T-cell
(2)
(1)
(2)
T-cell
Induction


Ag
S-
(1)
T-cell
(1)
Unresponsive

4.2 METHODS

4.2.1. *Culture conditions and preparation of cells.*

Cell suspensions were prepared as described in Sections 2.4 and 2.5 and cultures carried out as described in Sections 2.3 and 2.8. All experiments consisted of triplicate one ml cultures of appropriate dilutions of γ- or UV-irradiated stimulator cells (Section 2.6) and 10^6 responding C57Bl/6 or BALB/c lymph node cells in F-15 culture medium (Section 2.2).

4.2.2 *Preparation of CS.*

CS was prepared from C57Bl/6 ($H\text{-}2^b$), CBA ($H\text{-}2^k$) or C3H ($H\text{-}2^k$) spleen cells as described in Section 2.9.

4.2.3 *Cytotoxic assay.*

Triplicate wells were pooled and appropriate dilutions tested using the standard procedure described in Section 2.8. All cytotoxic activities are expressed as CU/culture.

4.2.4 *Nylon wool filtration of responding lymph node cells.*

This was carried out essentially as described by Julius *et al.* (1973). Individual 10 ml glass syringe barrels were packed up to the 6 ml mark with 0.6 g untreated nylon wool taken from LP-1 Leuko-Pak Leukocyte Filters (Fenwal Laboratories) and autoclaved. Columns were prepared for use by soaking the wool with 5 ml Hank's BSS and incubating at 37°C for 60 minutes prior to use. The Hank's BSS was then expelled from the column, replaced with 2 ml F-15 culture medium containing $1\text{-}2\times10^8$ lymph node cells, and the columns incubated for a further 45 minutes at 37°C. The columns were

then washed through with warm F-15 culture medium, the flow rate being controlled by a 22G needle attached to the syringe barrel. The first 24 ml of effluent were collected from each column, centrifuged, and resuspended in fresh F-15 culture medium.

4.3 RESULTS

4.3.1 *Stimulation characteristics of normal and oncogenic cells.*

Cytotoxic cell development in cultures of lymph node cells stimulated by oncogenic S^+ cells (Figure 4.3) displays similar kinetics to those described by Davidson (1977) for stimulation by normal spleen cells. Cytotoxic activity reaches a detectable level on the fourth day of culture, rises to a maximum by day 5, maintains this level through the sixth day of culture, and then declines. Unless otherwise stated all cytotoxic activities in the following studies were assayed on the fifth day of culture.

Figure 4.4 shows the stimulation characteristics of three tumour lines and two normal cell populations for allogeneic lymph node cells. Two of the tumour cells, P815 and EL-4, which are of mesenchymal origin, showed a stimulation profile similar to that of normal peritoneal cells. Thus, these two tumours express the S^+ phenotype of normal leucocytes. Spleen cells, in this system, are 10-100 fold less efficient as stimulators than either peritoneal or tumour cells. This may indicate that spleen cell populations contain fewer S^+ cells than the latter two populations. High doses of both normal and oncogenic cells are suppressive.

Figure 4.3 Cytotoxic responses of C57Bl/6 lymph node cells to γ-irradiated P815 in 1 ml cultures in F-15 culture medium under standard culture conditions (Section 2.3).

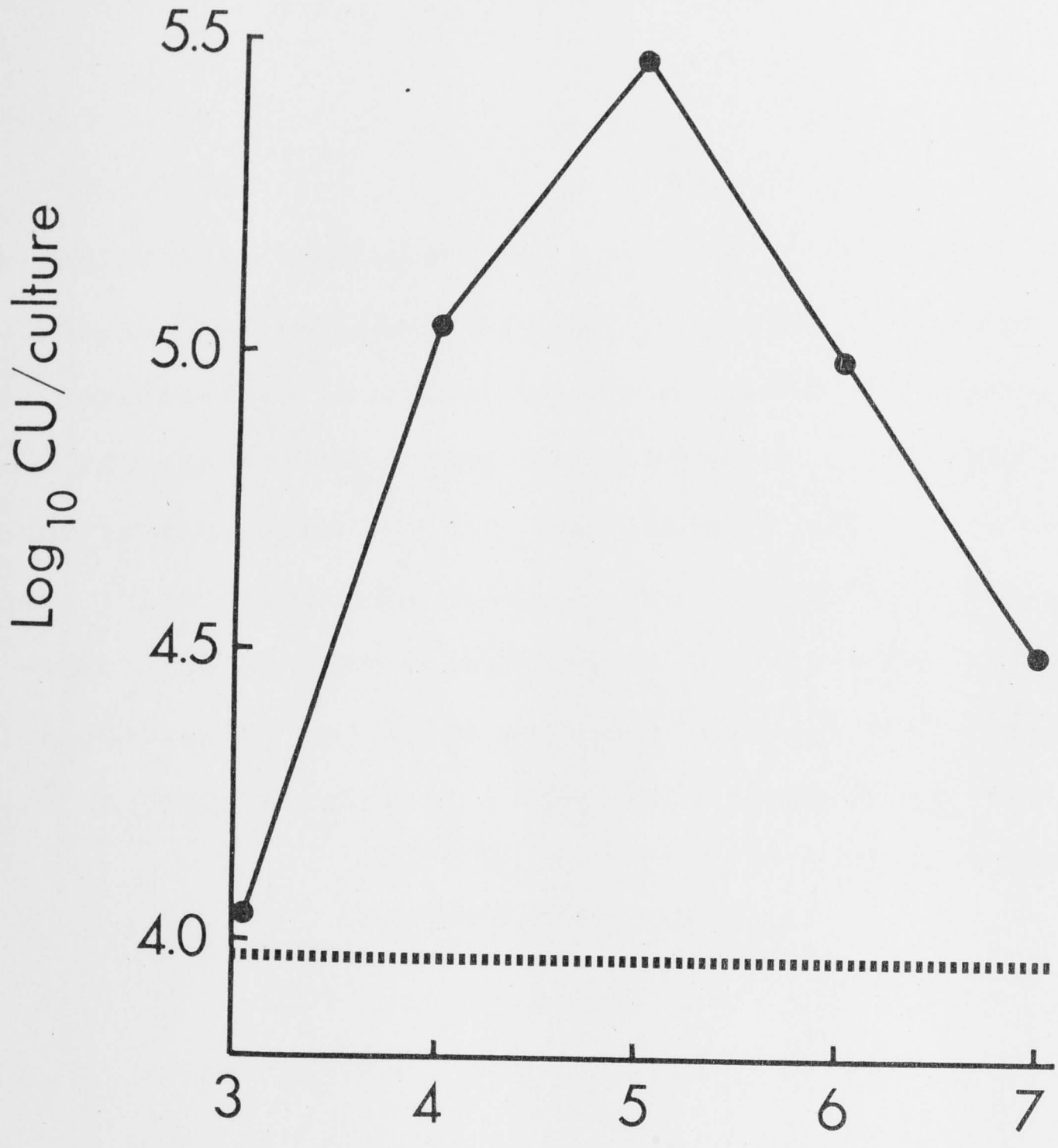
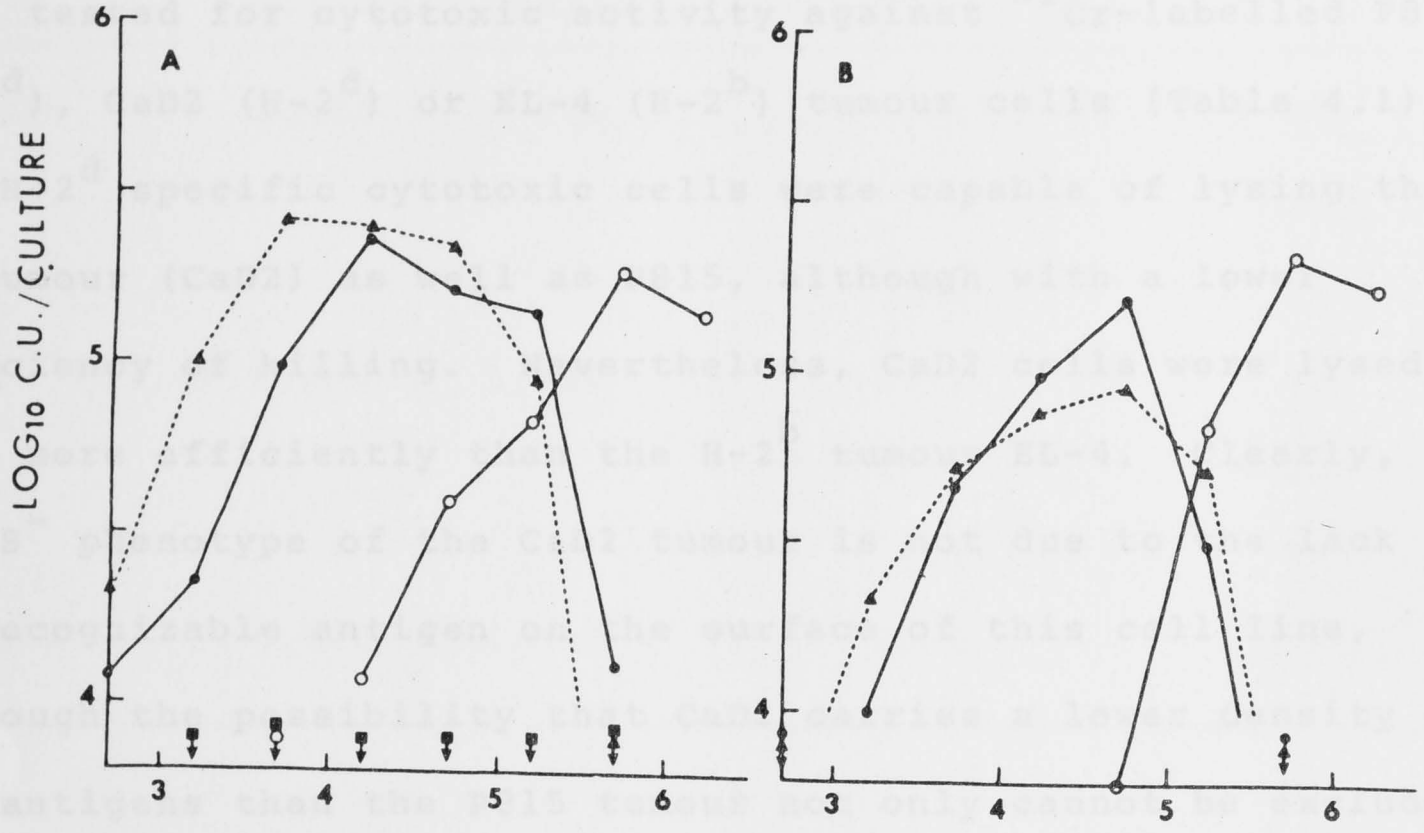

5.5
5.0
4.5
4.0
Log 10 CU/culture
3
4
5
6
7
Time in culture (Days)

Figure 4.4 (A) Cytotoxic cell responses of C57Bl/6 ($H\text{-}2^b$) lymph node cells cultured with the following $H\text{-}2^d$ cells: O, γ-irradiated (750 R) BALB/c spleen cells; ●, γ-irradiated (750 R) BALB/c peritoneal cells; ▲, γ-irradiated (5000 R) P815 mast tumour cells; ■, γ-irradiated (5000 R) CaD2 mammary tumour cells. (B) Cytotoxic cell response of BALB/c ($H\text{-}2^d$) lymph node cells cultured with the following $H\text{-}2^b$ cells: O, γ-irradiated (750 R) C57Bl/6 spleen cells; ●, γ-irradiated (750 R) C57Bl/6 peritoneal cells; ▲, γ-irradiated (5000 R) EL-4 lymphoma cells.


A
B
6
5
4
3
4
5
6
LOG10 C.U./CULTURE
LOG10 STIMULATING CELL DOSE

The third tumour, the DBA/2 mammary carcinoma (CaD2), shows no stimulator activity over the wide dose range tested and is therefore classified as an S^- cell line. The following experiment demonstrates that this failure of CaD2 to stimulate allogeneic lymphocytes is not due to a lack of recognizable $H\text{-}2^d$ antigens on the surface of this tumour line. C57Bl/6 lymphocytes were activated against the $H\text{-}2^d$ antigens on irradiated DBA/2 spleen cells. On day 5, the cultured cells were tested for cytotoxic activity against ^{51}Cr-labelled P815 ($H\text{-}2^d$), CaD2 ($H\text{-}2^d$) or EL-4 ($H\text{-}2^b$) tumour cells (Table 4.1). The $H\text{-}2^d$ specific cytotoxic cells were capable of lysing the S^- tumour (CaD2) as well as P815, although with a lower efficiency of killing. Nevertheless, CaD2 cells were lysed much more efficiently than the $H\text{-}2^b$ tumour EL-4. Clearly, the S^- phenotype of the CaD2 tumour is not due to the lack of recognizable antigen on the surface of this cell line, although the possibility that CaD2 carries a lower density of H-2 antigens than the P815 tumour not only cannot be excluded but is quite likely in the light of evidence suggesting that cells of haematogenous origin generally express larger amounts of histocompatibility antigens than somatic cells (Klein, 1975c). The failure of CaD2 cells to stimulate is also not due to their treatment with trypsin. Peritoneal cells treated in the same way retained their stimulatory capacity (Figure 4.5).

4.3.2 *UV-inactivation of S^+ cells.*

The S^+ phenotype of oncogenic cells can be destroyed by UV-radiation. Irradiation for 4 minutes with untraviolet light

TABLE 4.1

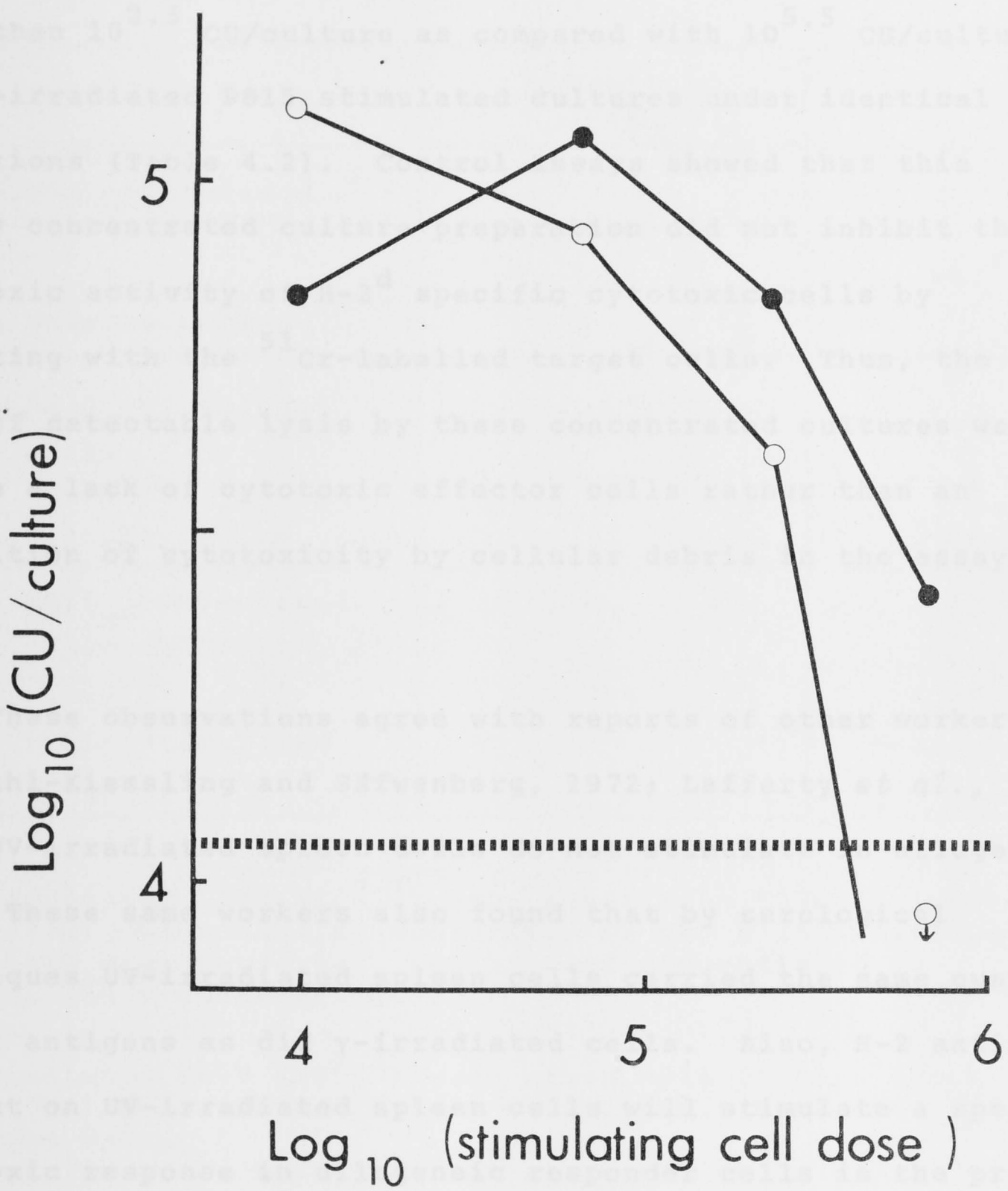
*Cytotoxic activity of H-2^d-reactive cells on P815, CaD2, and EL-4 target cells**

Target Cells	Lysis, cpm†		Cytotoxic/ Target cells	% specific ^{51}Cr release
	Water	Medium		
10^5P815	8,460	708	4:1	81
			2:1	44
			1:1	23
2 x 10^4	6,270	1,130	20:1	50
CaD2			10:1	32
			5:1	15
10^5EL-4	4,310	1,526	20:1	<5

* Prepared in 5-day cultures of 10^6 C57Bl/6 lymph node cells and 10^6 γ-irradiated DBA/2 spleen cells.

† ^{51}Cr-release from labelled target cells.

Figure 4.5 · Cytotoxic cell responses of C57Bl/6 lymph node cells cultured with various numbers of DBA/2 peritoneal cells. O, incubated 20 min with 0.1% trypsin and washed; ●, untreated. The broken line shows the response of lymph node cells alone cultured in the presence of CS.


5
4
Log 10 (CU/culture)
4
5
6
Log 10 (stimulating cell dose)

destroys the stimulatory capacity of P815 tumour cells at all doses tested. The cytotoxic acitivity of culutres stimulated with UV-inactivated P815 cells was tested using a 48-fold concentrated effector population in the cytotoxic assay. This experiment revealed that the activity of these cultures was less than $10^{2.5}$ CU/culture as compared with $10^{5.5}$ CU/culture for γ-irradiated P815 stimulated cultures under identical conditions (Table 4.2). Control assays showed that this highly concentrated culture preparation did not inhibit the cytotoxic activity of H-2^{d} specific cytotoxic cells by competing with the ^{51}Cr-labelled target cells. Thus, the lack of detectable lysis by these concentrated cultures was due to a lack of cytotoxic effector cells rather than an inhibition of cytotoxicity by cellular debris in the assay wells.

These observations agree with reports of other workers (Lindahl-Kiessling and Säfwenberg, 1972; Lafferty *et al.*, 1974) that UV-irradiated spleen cells do not stimulate in allogeneic MLC. These same workers also found that by serological techniques UV-irradiated spleen cells carried the same quantity of H-2 antigens as did γ-irradiated cells. Also, H-2 antigen present on UV-irradiated spleen cells will stimulate a specific cytotoxic response in allogeneic responder cells in the presence of an intact third party stimulator cell (Lafferty *et al.*, 1974; Bach *et al.*, 1977). This indicates that UV-irradiated cells not only retain the H-2-coded glycoprotein molecules on their surface but that these molecules are retained in a form suitable for the activation of cytotoxic T cells under standard culture conditions. In summary, UV-irradiation does not destroy

TABLE 4.2

Cytotoxic activity of C57Bl/6 lymph node cells stimulated with UV-irradiated P815 in vitro

Cells tested in assay	% Specific lysis	CU/culture
[1] 50µl $P815_{uv}$-stimulated Ln cells (x50) + 50µl culture medium	<5%	$<10^{2.5}$
[2] 50µl. $P815_{\gamma}$-stimulated Ln cells + 50µl culture medium	80%	$10^{5.7}$
50µl $P815_{uv}$-stimulated Ln cells (x50) + 50µl $P815_{\gamma}$-stimulated Ln cells	80%	$10^{5.7}$

[1] UV-irradiated P815 ($10^{5.2}$) were mixed with C57Bl/6 lymph node (ln) cells in 1 ml cultures. On day 5 25 individual cultures were harvested, pooled, and concentrated to 0.5 ml (50 x concentration).

[2] γ-irradiated P815 ($10^{5.2}$) were mixed with C57Bl/6 lymph node (Ln) cells in a single 1 ml culture. This was assayed on day 5.

50µl volumes of these cell suspensions were added to assay wells containing 10^5 ^{51}Cr-labelled P815 in 100 µl F-15 culture medium.

the antigenic determinants recognized by cytotoxic T cell precursors, but does inactivate the capacity of these cells to activate cytotoxic T cells.

4.3.3 *Con A-induced spleen cell supernatant (CS) facilitates T cell activation by S^- tumour cells in allogeneic MLC.*

According to the theory of allogeneic stimulation, we would expect T cell activation to occur if we could provide a source of the inductive second signal to cultures containing S^- tumour cells and allogeneic T cells. We might expect to obtain this factor from S^+ cells as such as the S^+ tumours or spleen cells. So far attempts to detect this activity in the supernatant medium from P815 or spleen cells have failed. However, when Con A is added to spleen cells and the cells are then cultured for 16 hours in the absence of serum (Section 2.9) an active supernatant is produced (CS) which contains an activity with the properties predicted for the inductive second signal. The factor alone does not cause cytotoxic T cell activation but will cause activation when added to cultures of S^- cells and allogeneic lymphocytes.

The addition of CS (10% by volume) to cultures of C57Bl/6 lymph node cells and either UV-irradiated P815 or γ-irradiated CaD2 cells facilitated full and specific cytotoxic responses to the H-2^d stimulator cells (Figures 4.6 and 4.7). Cells generated in this way demonstrated no detectable lysis ($<10^4$ CU/culture) on syngeneic (EL-4) or third-party (Rl, H-2^k) target cells. Control cultures containing lymph node cells alone in the presence of CS generated a detectable but low degree of cytotoxic activity against P815 targets. This

Figure 4.6 Cytotoxic cell response of C57Bl/6 lymph node cells, cultured with UV-irradiated (960 $\mu W/cm^2$ in the 230 to 270 nm range) P815 mast cell tumour cells: ●, response in the absence of CS; o, response in the presence of CS. The broken line shows the response of lymph node cells alone cultured in the presence of CS.

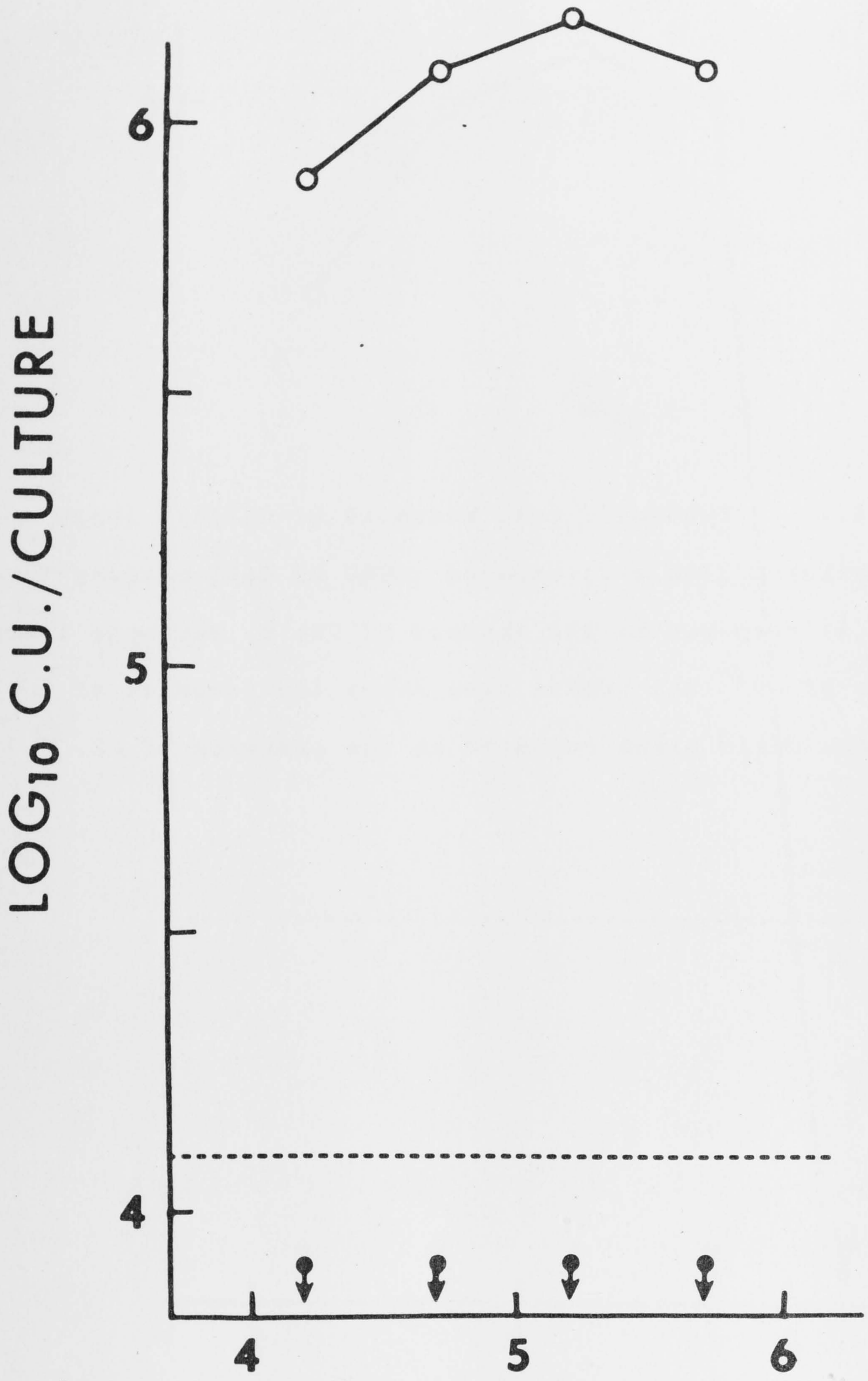
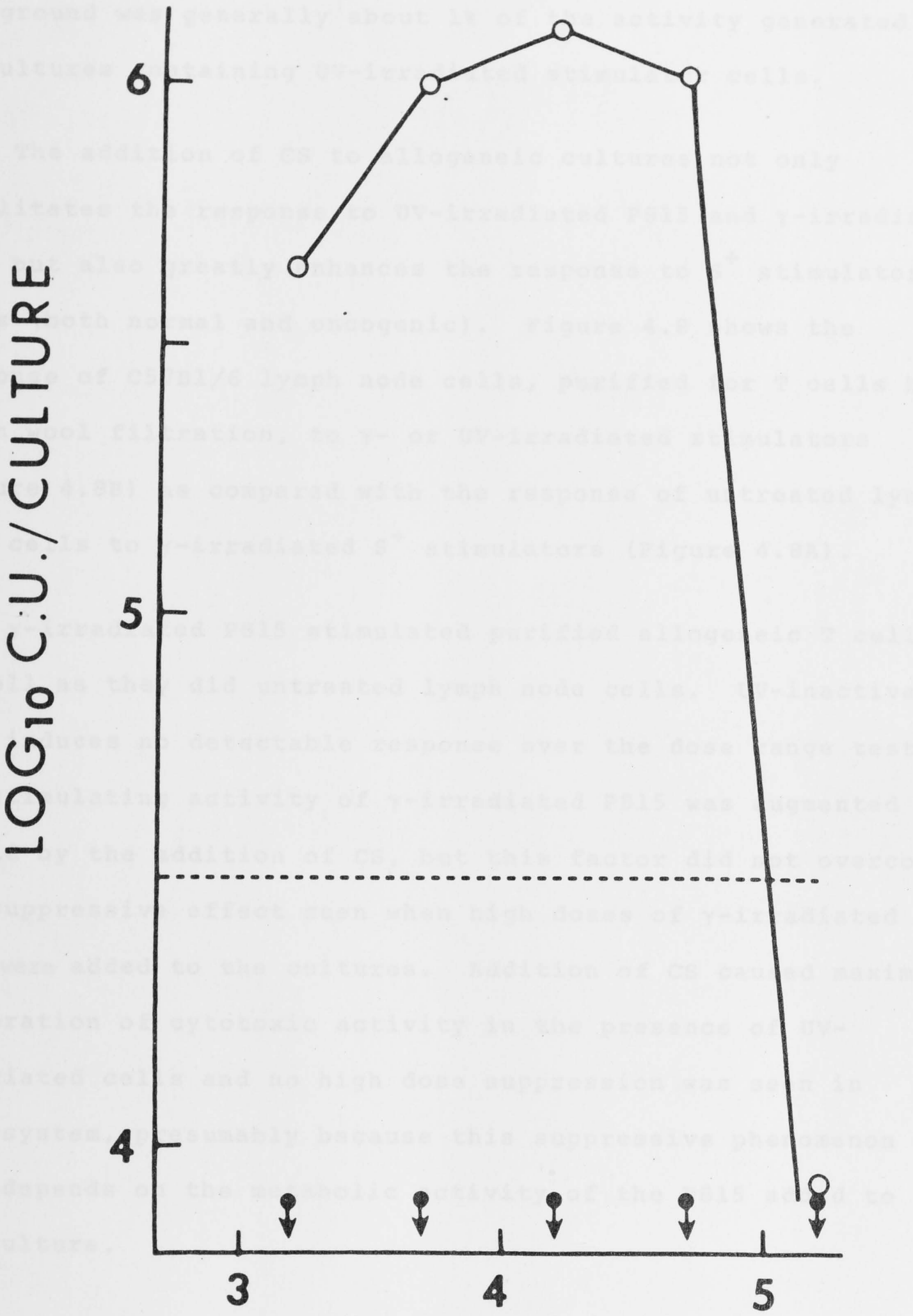

LOG10 C.U./CULTURE
6
5
4
4
5
6
LOG10 STIMULATING CELL DOSE

Figure 4.7 Cytotoxic cell response of C57Bl/6 lymph node cells cultured with γ-irradiated (5000 R) CaD2 mammary tumour cells: •, response in the absence of CS; o, response in the presence of CS. The broken line shows the response of lymph node cells alone cultured in the presence of CS.


6
5
4
3
4
5
LOG10 C.U./CULTURE
LOG10 STIMULATING CELL DOSE

background was generally about 1% of the activity generated in cultures containing UV-irradiated stimulator cells.

The addition of CS to allogeneic cultures not only facilitates the response to UV-irradiated P815 and γ-irradiated CaD2 but also greatly enhances the response to S^+ stimulator cells (both normal and oncogenic). Figure 4.8 shows the response of C57Bl/6 lymph node cells, purified for T cells by nylon wool filtration, to γ- or UV-irradiated stimulators (Figure 4.8B) as compared with the response of untreated lymph node cells to γ-irradiated S^+ stimulators (Figure 4.8A).

γ-irradiated P815 stimulated purified allogeneic T cells as well as they did untreated lymph node cells. UV-inactivated P815 induces no detectable response over the dose range tested. The stimulating activity of γ-irradiated P815 was augmented 5-fold by the addition of CS, but this factor did not overcome the suppressive effect seen when high doses of γ-irradiated P815 were added to the cultures. Addition of CS caused maximal restoration of cytotoxic activity in the presence of UV-irradiated cells and no high dose suppression was seen in this system, presumably because this suppressive phenomenon also depends on the metabolic activity of the P815 added to the culture.

These data demonstrate that cytotoxic T cells (T_C) can be activated when UV-irradiated cells are presented to responsive cells in the absence of accessory cells such as macrophages but in the presence of CS. Similarly, purified T_C cells can be activated by γ-irradiated tumour cells in the absence of both accessory cells and CS.

Figure 4.8 Cytotoxic cell response of C57Bl/6 (H-2^b) lymph node cells (10^6/culture).

A. Normal lymph node cells responding to

Δ——Δ γ-irradiated (750 R) BALB/c (H-2^d) spleen cells

▲——▲ γ-irradiated (750 R) BALB/c peritoneal cells

O——O γ-irradiated (5,000 R) P815 (H-2^d) tumour cells

B. Column-purified T cells responding to

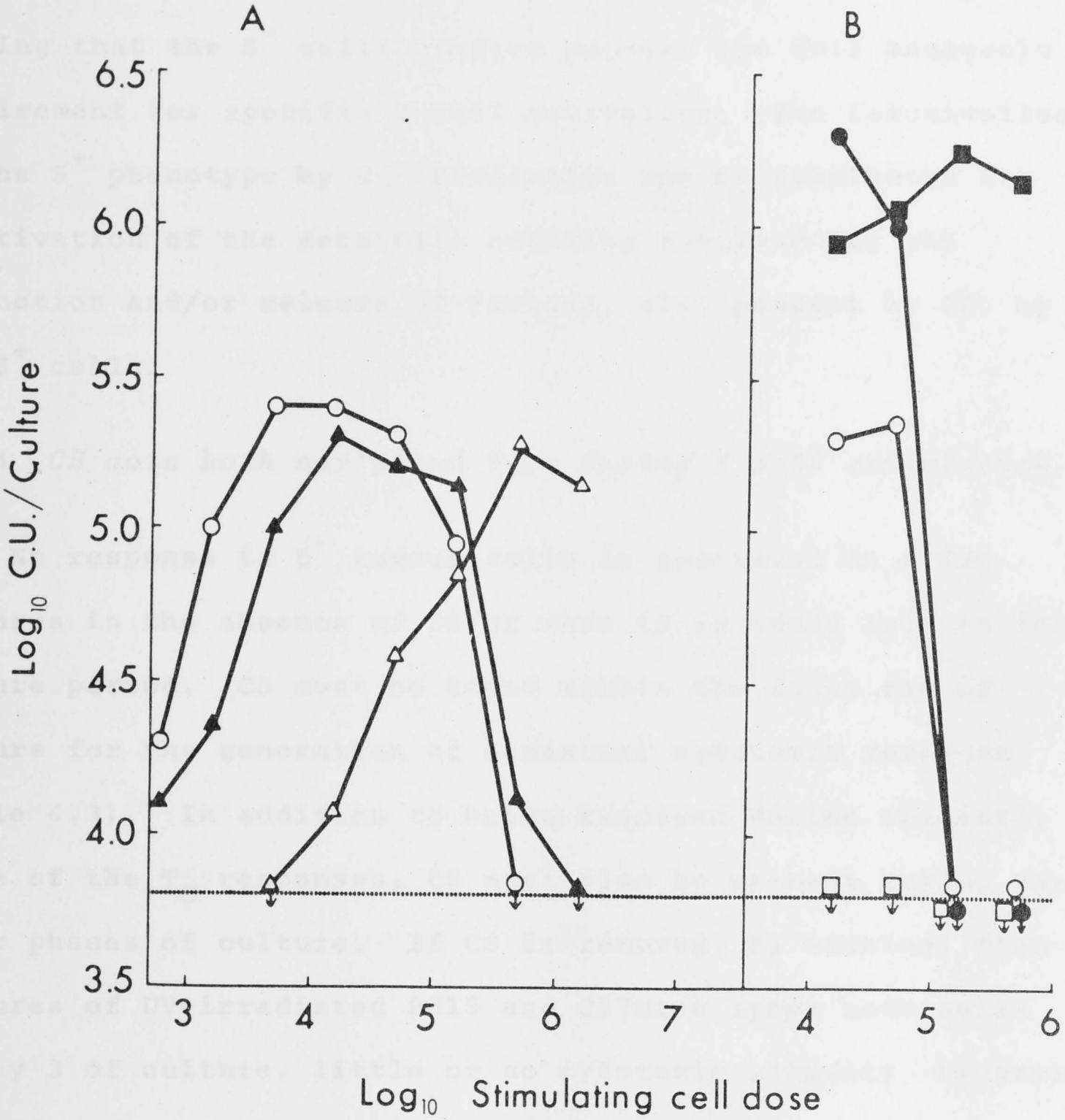
O——O γ-irradiated (5,000 R) P815 tumour cells.

●——● γ-irradiated (5,000 R) P815 tumour cells + CS (10%)

□——□ UV-irradiated P815.

■——■ UV-irradiated P815 + CS (10%)

The horizontal broken line shows the lower level of assay sensitivity.


A
B
6.5
6.0
5.5
5.0
4.5
4.0
3.5
Log10 C.U./Culture
3
4
5
6
7
4
5
6
Log10 Stimulating cell dose

We can conclude from these studies that transplantation antigen, presented on the surface of S^- cells is not a sufficient requirement for allogeneic lymphocyte activation. In the presence of CS, specific T cell activation does occur, showing that the S^- cells studied possess the full antigenic requirement for specific T cell activation. The inactivation of the S^+ phenotype by UV-irradiation can be attributed to inactivation of the metabolic activity required for the production and/or release of factors, also present in CS, by the S^+ cells.

4.3.4 *.CS acts both early and late during T cell stimulation.*

No response to S^- tumour cells is generated in 5 day cultures in the absence of CS or when CS is added late in the culture period. CS must be added within the first day of culture for the generation of a maximal cytotoxic response (Table 4.3). In addition to being required during the early phase of the T_C responses, CS must also be present during the later phases of culture. If CS is removed, by washing, from cultures of UV-irradiated P815 and C57Bl/6 lymph node cells on day 3 of culture, little or no cytotoxic activity is present on day 5 (Table 4.4).

Thus, CS must be present in the culture medium at or around the same time as the addition of antigen. That is, the factors present in CS act early. Furthermore, CS must remain in the culture medium throughout the 5 day culture period for the ultimate expression of T_C activity.

TABLE 4.3

Cytotoxic response of C57B1/6 lymph node cells (LNC) to UV-treated CaD2 and P815 cells following additions of CS at various times

Cells cultured X 10^{-5}			Log_{10} (CU*/culture) on day 5 with CS added on day					
C57B1/6 LNC	UV P815	UV CaD2	0	1	2	3	4	Not added
10	1.6	-	5.7†	5.6	<3.7	<3.7	NT	<3.7
10	-	1.6	6.2	5.9	4.4	<3.9	<3.9	<3.9
10	-	-	4.6	NT	<3.9	NT	NT	<3.9

NT = not tested.

* When $^{3}/_{10}$ of a culture is assayed, 6 log_{10} CU is equivalent to 95% specific lysis; 5 log_{10} CU to 26% lysis; and 4 log_{10} CU to 3% lysis.

† Cytotoxic activity in similar cultures was destroyed by anti-Θ serum plus complement. Other cultures were not so tested.

TABLE 4.4

Costimulator Requirement During Culture Period for Cytotoxic Cell Activation

REACTION MIXTURE[1]	CS PRESENT[2] DAY 0-3	CS PRESENT[2] DAY 3-5	LOG_{10} CU/CULTURE ON DAY 5
$P815_{(UV)}$ + C57Bl/6 lymph node	+	+	6.0
	+	-	3.9
	-	+	<3.9
	-	-	<3.9

1. 0.5 ml of tumour cells ($10^{5.5}$/ml) mixed with 0.5 ml of lymph node cells (2 x 10^6/ml) in F-15 culture medium.

2. 0.1 ml of costimulator preparation added to 1 ml cultures.

CU, cytotoxic units; CS, Con A-stimulated spleen cell supernatant.

4.3.5 *CS is not strain specific*

CS preparations generated from CBA, C3H, or C57Bl/6 spleen cells were tested for activity in cultures of UV-irradiated P815 or CaD2 (10^5 cells/culture) and C57Bl/6 lymph node cells (10^6 cells/culture). Each preparation of CS was tested at three dilutions. The results of this experiment (Table 4.5) indicate that there is no strain specificity associated with the activity of this factor.

4.4 DISCUSSION

The capacity of tumour cells to activate allogeneic cytotoxic cells *in vitro* is dependent on both the nature of the tumour cell and its metabolic activity. In this study tumour lines of mesenchymal origin (the EL-4 lymphoma and the P815 mastocytoma) showed stimulating activity. These tumours, following γ-irradiation to prevent cell proliferation, had stimulation characteristics that were similar to those of γ-irradiated peritoneal cells obtained from mice of the same H-2 haplotype. The stimulation profile of both normal and oncogenic stimulator cells passed through a maximum, and a marked suppression of the cytotoxic cell reponse was seen at high stimulator cell densities. In the case of peritoneal cells, this suppression has been shown to result from the production of arginase by the peritoneal cells, which depletes the medium of an essential amino acid (arginine) required for lymphocyte differentiation (Kung *et al.*, 1977). No high dose suppression by UV-irradiated P815 was observed indicating that these tumour cells, too, probably exert their suppressive effects via the release of metabolites during culture.

TABLE 4.5

Strain specificity of CS

Source of CS	Dilution of CS in culture	Log_{10} (CU/culture) with various stimulating cells*		
		10^5 UV-P815	10^5 UV-CaD2	None
C57Bl/6 spleen	1:10	6.1	6.3	4.4
	1:30	5.5	5.3	<3.9
	1:100	5.1	4.9	<3.9
CBA spleen†	1:10	6.1	5.9	4.9
	1:30	5.4	5.0	4.1
	1:100	4.6	4.5	<3.9
C3H spleen	1:10	6.3	6.2	4.7
	1:30	NT	5.4	4.2
	1:100	NT	4.7	<3.9
None added	-	<3.9	<3.9	<3.9

NT = not tested.

* Responding cells were 10^6 C57Bl/6 lymph node cells, the target cells were ^{51}Cr-labelled P815 cells.

† Specific lysis of labelled EL-4 cells was less than 3% (3.9 log_{10} CU) in all tests with CBA CS.

The epithelial tumour, CaD2, showed no stimulating activity when tested over a wide dose range. This tumour was specifically lysed by cytotoxic cells activated to the H-2^d antigens of the DBA/2 mouse strain, indicating that the failure of this cell line to stimulate was not due to a lack of recognizable antigen on the surface of this cell. Tumour cell lines can therefore be classified as S^+ or S^- on the basis of their capacity to activate allogeneic cytotoxic cells *in vitro*. The S^+ phenotype of the P815 tumour was inactivated by UV-irradiation, and, in this respect, these cells behaved similarly to normal lymphoid cells (Lafferty *et al.*, 1974).

The addition of CS to cultures of S^- cells (either γ-irradiated CaD2 or UV-inactivated P815) and allogeneic (C57Bl/6) lymph node cells resulted in the generation of a specific cytotoxic cell response to H-2 antigens on the surface of the S^- cells. These S^- cells therefore possess the antigen required for recognition by allogeneic lymphocytes but lack a further requirement for T cell activation that is provided by the CS. C57Bl/6 lymphocytes cultured alone in the presence of CS showed a low but significant level of cytotoxic activity against P815 target cells. These tumour cells were maintained *in vitro* in medium supplemented with 10% FCS, and it is possible that this low level of cytotoxicity may represent a response to calf serum proteins present in the lymphocyte culture medium. Preliminary data from our laboratory supports this hypothesis. in that this background response is not detected on targets maintained in normal mouse serum rather than FCS (Warren, personal communication).

Our interpretation of these experimental findings is that T cell activation has a two-signal triggering mechanism (Figures 4.1 and 4.2) and that S^+ cells provide both antigen (signal 1) and a source of the second non-specific signal required for T cell activation; either signal alone is insufficient for T cell activation as suggested by Lafferty and Cunningham (1975). According to this model, UV-irradiation of S^+ cells would convert them to the S^- phenotype by inactivating those metabolic activities required for the production and/or release of the second signal by these cells. CS would provide an exogenous source of the second signal. The CaD2 tumour intrinsically lacks the metabolic activities required for the production of the second signal and are therefore S^-.

Paetkau *et al.*, (1976) have postulated that CS provides a *costimulator* for the mitogenic activation of thymocytes by Con A. We propose that the same factor (costimulator) provides the inductive second signal in our model. The finding that CS has an early effect, i.e., must be present in cultures during the early phase of antigen presentation by S^- cells (day 0 or day 1 of culture) is consistent with this idea. Under conditions where the antigen-presenting cells have intrinsic stimulating activity (S^+) CS acts as an amplifier of the cytotoxic cell response. This amplification of the intrinsic response was observed when CS was added to cultures as late as the third day of culture. At this stage, we cannot say whether the lymphocyte costimulator and amplifier factors are the same or different molecules present in the CS preparation. This aspect of CS activity will be discussed further in Chapter 6.

The activity of CS in these experiments cannot be due to contamination with Con A. All of the Con A that was not cell bound was removed from the spleen cultures by three washes after the initial 2 hour culture. Con A could not be detected in the final CS preparation by erythrocyte agglutination, nor could activity be removed from CS on a Sephadex G-10 column. Further evidence that the activity of CS is not due to Con A is that CS activity is highly species specific in its action. This evidence will be discussed in Chapter 5.

Bach *et al* (1976, 1977) have postulated that the second signal required for T_C activation is provided by a distinct class of non-cytotoxic helper T cells (T_H) which are stimulated predominantly by I-region coded determinants on the stimulator cell. There is, however, considerable evidence against this hypothesis. Cytotoxic T cell activation can occur in cultures containing responding cells which differ from the stimulating cells at only a single genetic locus within the H-2K or H-2D genetic regions (Berke and Amos, 1973; Forman and Klein, 1975; Melief *et al.*, 1977; Nabholz *et al.*, 1975; Widmer *et al.*, 1973). The stimulatory capacity of cell populations is not related to the presence of Ia antigens on their surface. Both EL-4 and P815 are efficient stimulators of T_C cell responses *in vitro* but do not express detectable levels of Ia antigens (Cullen *et al.*, 1974; Frelinger *et al.*, 1974). Paetkau *et al* (1976) have shown that the non-specific activation of T cells by Con A also requires CS activity. Our own studies have indicated that both H-2K/D and H-2I region stimulation generates, predominantly, clones of cytotoxic T cells arguing against the existence of a distinct class of non-cytotoxic helper T cells. This evidence

will be discussed in Chapter 7. Hence, there is no evidence for the existence of a distinct class of non-cytotoxic helper T cells.

Our conclusions from these studies can be summarized as follows:

1. Tumour cell lines can be classified according to their stimulatory capacity (S^+ or S^-). Both S^+ and S^- cells express histocompatibility antigens which can be recognized by cytotoxic T cell precursors and effector cells.

2. UV-irradiation destroys the stimulatory capacity of S^+ cells, rendering them S^-, without destroying their histocompatibility antigens.

3. All S^- cells, whether intrinsically S^- or rendered S^- by UV-irradiation, behave as S^+ cells in the presence of CS.

4. CS acts early and is required continuously throughout the response.

5. CS is not strain specific.

6. Adherent or immunoglobulin-bearing accessory cells are not required for the activation of cytotoxic T cells either in the presence or absence of CS.

We postulate that immune induction of T cells occurs when the responsive cell binds antigen through its surface receptor (signal 1) and simultaneously receives an inductive stimulus (signal 2) from the stimulator cell. We define this inductive second signal as the *lymphocyte costimulator*.

In this light our findings that CS is required both early and late during T cell activation can be interpreted in several ways. There may be two activities in CS that are required for T_C activation, an early acting 'costimulator' and a late acting maintenance or amplifier activity. In this case costimulator would be required for the initial induction of T_C effector cells while the latter would be required for the later proliferative phase of the response. A second alternative is that 'costimulator' acts by interfering with an intrinsic 'switch off' mechanism that is operative in the responding T cells. In this case costimulator would be required early and its removal at any stage would abort the response. Studies which have yielded some answers with respect to this question will be discussed in Chapter 6.

4.5 SUMMARY

In vitro T cell activation requires both antigen presentation and a second stimulus provided by the lymphocyte costimulator. Neither alone is sufficient to induce specific T cell activation. The S^+ phenotype of stimulating cells is dependent on the metabolic activity of these cells. This finding is consistent with the notion that production and/or release of the costimulator is a function of metabolically active cells. The costimulator acts at an early stage of the interaction between lymphocyte and antigen, and the costimulator, or a separate maintenance factor, is required throughout the culture period for the expression of full cytotoxic activity. The lymphocyte costimulator is not strain specific.

CHAPTER FIVE

PROLIFERATIVE AND CYTOTOXIC RESPONSES TO ALLOGENEIC AND XENOGENEIC ANTIGENS *IN VITRO*

5.1 INTRODUCTION

Reactivity in mixed leucocyte culture (MLC) has been studied as an *in vitro* model of the allograft reaction (Häyry and Defendi, 1970). Both reactivities involve cell-mediated responses to antigenic specificities coded by the major histocompatibility complex (MHC) of the species. Early attempts to explain the phenomenon of graft rejection were based on the concept that antigen alone drives the differentiation of immunocyte clones (Medawar, 1963). However, reports that MLC responses were not obtained with mixtures of xenogeneic leucocytes (Lafferty and Jones, 1969; Wilson and Nowell, 1970; Wilson and Fox, 1971; Greineder and Rosenthal, 1975) were not compatible with this concept since foreign antigens are clearly present on xenogeneic cells and xenograft reactions *in vivo* are particularly strong (Russell and Monaco, 1965). Other reports demonstrating normal responses in xenogeneic MLC (Asantila *et al.*, 1974; Nielson, 1972; Shons *et al.*, 1973; Widmer and Bach, 1972; Lindahl and Bach, 1975, 1976; Peck *et al.*, 1976) were in conflict with the initial negative reports, and recently Klein (1975b) dismissed the postulated lack of xenoreactivity as "a myth prevailing among immunologists ...".

In this report I present further evidence that xenogeneic MLC responses can be weak or non-existent, and attempt to rationalize the conflicting reports found in the literature.

5.2 METHODS

5.2.1 *Animals*

Male animals of the C57Bl/6 (H-2^b), CBA/H (H-2^k), DBA/2 (H-2^d) and SJL (H-2^s) mouse strains, BH, Le and DA rat strains,

outbred guinea pigs and randomly bred Merino sheep, goats, and Friesian cattle were used (Section 2.1).

5.2.2 *Cell culture techniques*

Leucocyte suspensions were prepared as described (Sections 2.4 and 2.5).

Mixed cell cultures for the generation of MLR were set up and assayed as described in Section 2.7 in 0.2 ml cultures.

Cytotoxic cell responses were generated in 1 ml cultures and assayed as described in Section 2.8.

5.2.3 *Preparation of CS*

CS was prepared from mouse (CBA/H), rat (Le), guinea pig, or bovine spleen cells or human PBL as described in Section 2.9. CS was generally used in cultures at a concentration of 10% v/v.

5.2.4 *Limiting dilution assay*

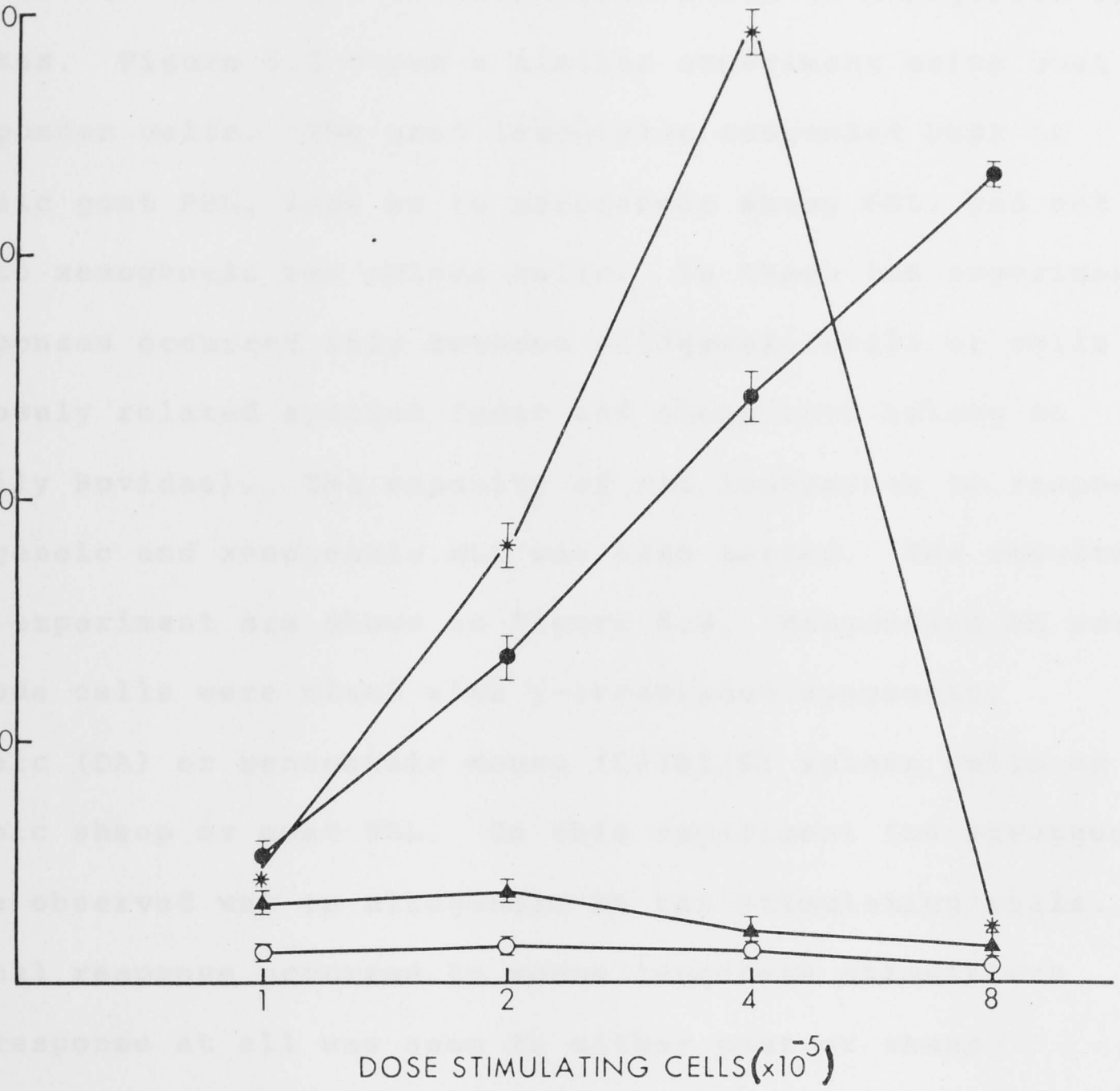
This was carried out as described in Section 2.10. All cultures were carried out in medium containing CS homologous with the responding cells at a concentration of 10% v/v. That is, guinea pig CS was used in cultures containing guinea pig responding cells and CBA/H CS was used in all mouse cultures.

5.3 RESULTS

5.3.1 *MLC responses in xenogeneic cultures.*

We have examined the proliferative MLC responses in three xenogeneic systems. Figure 5.1 shows the response of sheep peripheral blood leucocytes (PBL) to γ-irradiated autologous,

Figure 5.1 Response of sheep PBL (10^6) to different doses of γ-irradiated autologous sheep PBL (o-o), allogeneic sheep PBL (●-●), goat PBL (⋆-⋆) or rat spleen cells (▲-▲) in 0.2 ml cultures. ^{3}H-thymidine-uptake was measured on day 6 of culture as described (Section 2.7) and is expressed as C.P.M./$2x10^5$ cells.


40
30
20
10
C.P.M./2x10^5 CELLS(x10^-3)
1
2
4
8
DOSE STIMULATING CELLS(x10^-5)

allogeneic, or xenogeneic (goat PBL, or rat spleen) cells. The sheep PBL responded to allogeneic sheep or xenogeneic goat leucocytes but not at all to autologous sheep or xenogeneic rat leucocytes. Figure 5.2 shows a similar experiment using goat PBL responder cells. The goat leucocytes responded best to allogeneic goat PBL, less so to xenogeneic sheep PBL, and not at all to xenogeneic rat spleen cells. In these two experiments MLC responses occurred only between allogeneic cells or cells from closely related species (goat and sheep both belong to the Family Bovidae). The capacity of rat leucocytes to respond in allogeneic and xenogeneic MLC was also tested. The results of this experiment are shown in Figure 5.3. Responding BH rat lymph node cells were mixed with γ-irradiated syngeneic, allogeneic (DA) or xenogeneic mouse (C57Bl/6) spleen cells or xenogeneic sheep or goat PBL. In this experiment the strongest response observed was to allogeneic DA rat stimulating cells. A marginal response occurred to mouse leucocyte stimulators but no response at all was seen to either goat or sheep leucocytes. Again, the only response seen to xenogeneic cells was between closely related species (rat and mouse both belong to the Family Muridae).

5.3.2 *Cytotoxic cell responses to allogeneic and xenogeneic antigens.*

In a theoretical discussion of allogeneic interactions Lafferty and Cunningham (1975) postulated that T cell activation was a two signal process, requiring the provision of both antigen and an inductive second signal by the stimulating cell, and that the species specificity of these reactions resulted from the species specificity of the inductive signal. There is

Figure 5.2 Response of goat PBL (10^6) to different doses of γ-irradiated autologous goat PBL (o-o), allogeneic goat PBL (●-●), sheep PBL (⋆-⋆)or rat spleen cells (▲-▲) in 0.2 ml cultures. ^{3}H-thymidine uptake was measured on day 6 of culture as described (Section 2.7) and is expressed as C.P.M./2x10^5 cells.

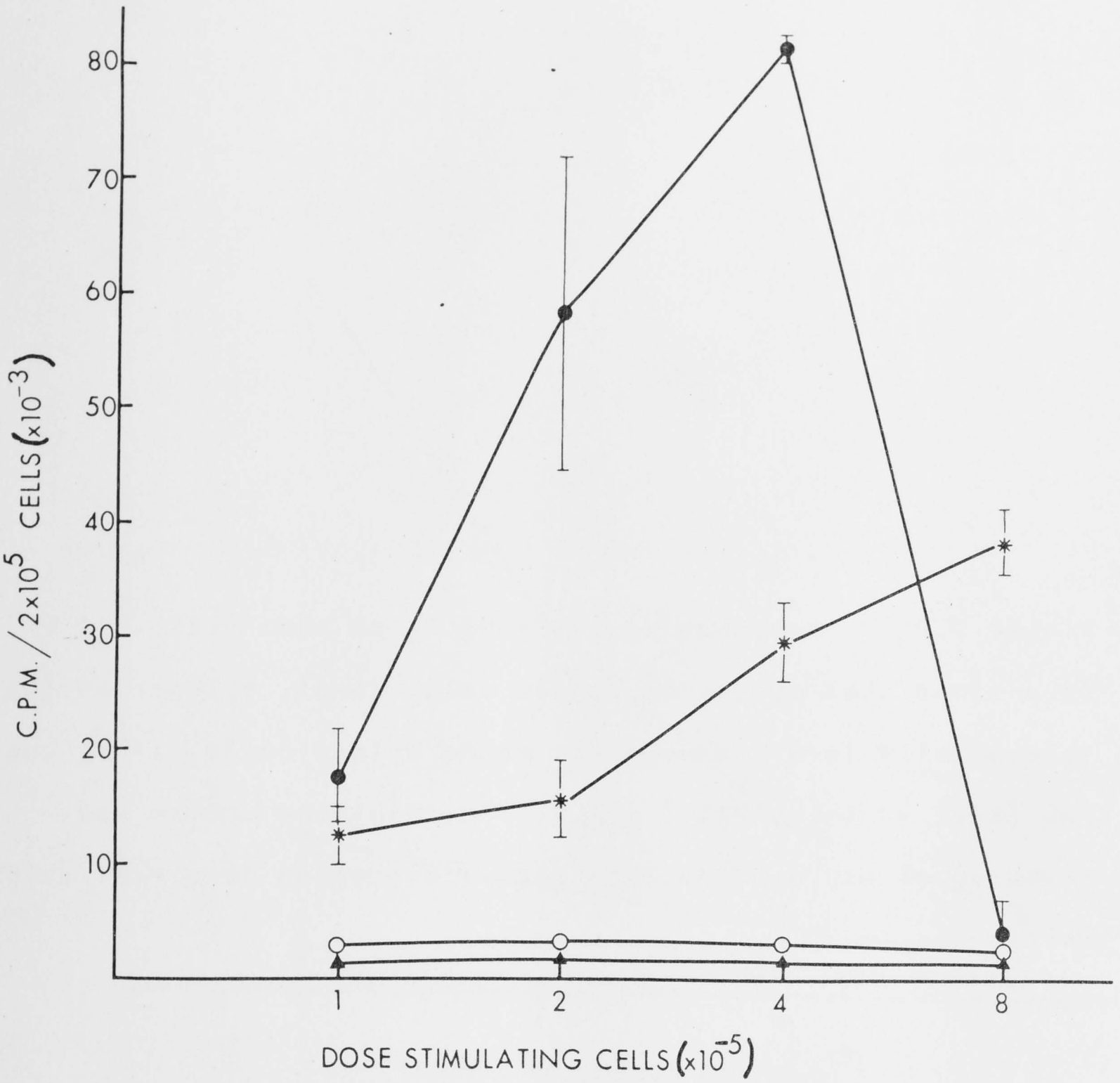
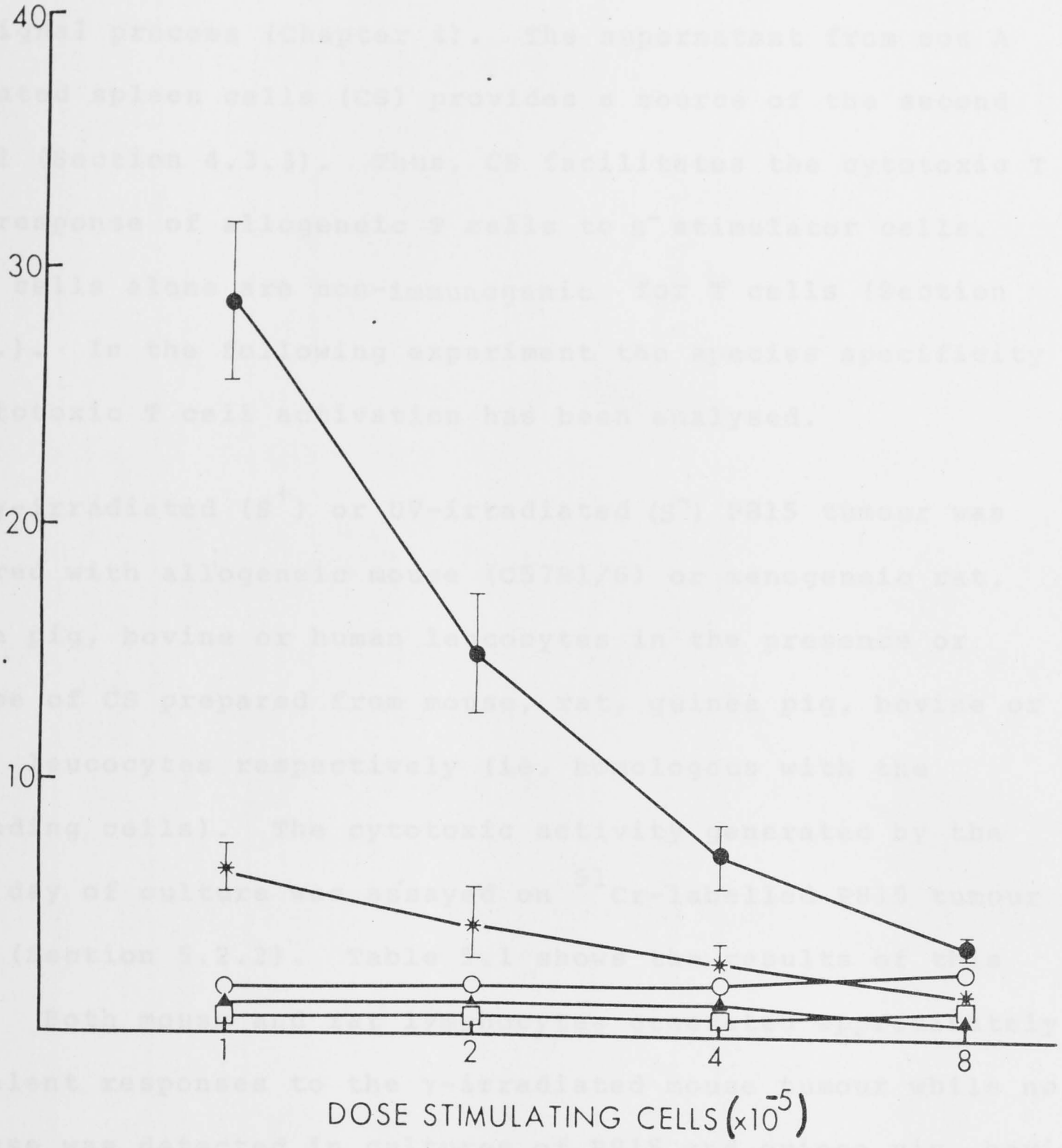

C.P.M./2x10^5 CELLS (x10^-3)
80
70
60
50
40
30
20
10
1
2
4
8
DOSE STIMULATING CELLS (x10^-5)

Figure 5.3 . Response of rat (BH) lymph node cells (10^6) to γ-irradiated syngeneic spleen cells (o-o), allogeneic (DA) spleen cells (●-●), xenogeneic mouse spleen cells (*-*), goat PBL (▲-▲) or sheep PBL (□-□). ^{3}H-thymidine uptake was measured on day 6 of culture and is expressed as C.P.M./$2x10^5$ cells.


40
30
20
10
C.P.M./2x10^5 CELLS (x10^-3)
1
2
4
8
DOSE STIMULATING CELLS (x10^-5)

now evidence indicating that *in vitro* T cell activation is a two signal process (Chapter 4). The supernatant from con A activated spleen cells (CS) provides a source of the second signal (Section 4.3.3). Thus, CS facilitates the cytotoxic T cell response of allogeneic T cells to S^- stimulator cells. These cells alone are non-immunogenic for T cells (Section 4.3.2.). In the following experiment the species specificity of cytotoxic T cell activation has been analysed.

γ-irradiated (S^+) or UV-irradiated (S^-) P815 tumour was cultured with allogeneic mouse (C57Bl/6) or xenogeneic rat, guinea pig, bovine or human leucocytes in the presence or absence of CS prepared from mouse, rat, guinea pig, bovine or human leucocytes respectively (ie. homologous with the responding cells). The cytotoxic activity generated by the fifth day of culture was assayed on ^{51}Cr-labelled P815 tumour cells (Section 5.2.2). Table 5.1 shows the results of this study. Both mouse and rat lymphocytes generated approximately equivalent responses to the γ-irradiated mouse tumour while no response was detected in cultures of P815 and guinea pig, bovine or human leucocytes. Mouse cells were unresponsive to the UV-inactivated mouse tumour, and the response of these cells was restored by addition to the cultures of mouse or rat CS, but not guinea pig, bovine or human CS. Guinea pig, bovine and human CS, however, were each effective in facilitating the response of homologous leucocytes to the γ-irradiated mouse tumour.

The cytotoxic response of guinea pig cells to the P815 tumour, induced in thepresence of guinea pig CS, was specific

TABLE 5.1

Phylogenetic Specificity of Costimulator

	Responding[1] cell	Treatment of P815	Log_{10} CU/Culture with Various CS Preparations[2]					
			None	Mouse	Rat	Guinea Pig	Bovine	Human
A.	Mouse L.N.	U V	<3.9	5.8	6.2	<3.9	<3.9	<3.9
	Guinea Pig L.N	γ-Ray	<3.9	<3.7	NT	5.3	NT	NT
	Bovine L.N.	γ-Ray	<3.9	<3.9	NT	NT	4.7	NT
B.	Human P.B.L.	γ-Ray	<3.9	<3.9	NT	NT	NT	4.7
	Mouse L.N.	γ-Ray	5.5	NT	NT	NT	NT	NT
	Rat L.N.	γ-Ray	5.2	NT	NT	NT	NT	NT

1 Reaction mixtures consisted of 0.5 ml of P815 tumour cells ($10^{5.5}$/ml) mixed with 0.5 ml of the responding lymphocyte preparation (2×10^6/ml)

2 0.1 ml of CS added to 1 ml cultures.

CU, cytotoxic units

L.N., lymph node cells

P.B.L., peripheral blood leucocytes.

for this cell and did not involve a detectable response to antigens common to different mouse strains (Table 5.2).

5.3.3 *Precursor frequency of P815-reactive cytotoxic cells in guinea pig lymph node cells.*

CS homologous to the responding lymphocytes expands cytotoxic T cell clones, and can be used for limiting dilution clonal analysis of cytotoxic T cell populations. This assay system is described in Section 2.10 and was used to estimate the precursor frequency of P815-reactive cytotoxic cells in ginea pig lymph node cell populations. Cultures containing γ-irradiated P815 and limiting dilutions of responder lymphocytes were set up in culture medium supplemented with guinea pig CS. The proportion of cytotoxic wells at different dilutions of responding lymphocytes was determined after a 7 day incubation period. Figure 5.4 shows the distribution of negative wells for this xenogeneic response to mouse antigens. On the basis of these data the frequency of P815-reactive cytotoxic precursors in guinea pig lymph node cells was 0.5×10^{-3}. This precursor frequency is within the range of precursor frequencies found for mouse lymphocytes responding to alloantigen (Table 5.3; Lindahl and Wilson, 1977).

5.4 DISCUSSION

The above results confirm the initial report that MLC reactions can be species specific (Lafferty and Jones, 1969). This species specificity is not absolute. Strong proliferative reactions were obtained with allogeneic cells. Xenogeneic cells also respond, but only when obtained from animals phylogenetically related to the stimulating cell donor. Thus, goat and sheep

TABLE 5.2

Specificity of cytotoxic cells generated in cultures of guinea pig lymph node cells, γ-irradiated P815 and guinea pig CS

Stimulating cultures		Log_{10} CU/culture against† ^{51}Cr-labelled targets	
Responders	Stimulators	P815	EL-4
Guinea pig lymph node	P815[1]	5.4	<4.2
BALB/c lymph node	EL-4[2]	<4.0	5.4

[1] Guinea pig lymph node cells (10^6) were cultured with γ-irradiated P815 ($10^{5.2}$) in 1 ml F-15 culture medium containing 10% CS made from guinea pig spleen cells as described in Section 5.2.3.

[2] BALB/c lymph node cells (10^6) were cultured with γ-irradiated EL-4 cells ($10^{5.2}$) in 1 ml F-15 culture medium in the absence of CS.

†Cytotoxic activity was estimated using the ^{51}Cr-release assay described in Section 2.8. Cytotoxic activity is expressed as log_{10} CU/culture as described in Chapter 3.

Figure 5.4 Distribution of negative wells in limit dilution cultures containing $10^{4.5}$ γ-irradiated P815 tumour cells and limiting dilutions of guinea pig lymph node cells. Individual wells contained 0.2 ml F-15 culture medium containing 10% guinea pig CS (v/v). On day 4, 0.1 ml volumes of supernatant were removed from each well and replaced with 0.1 ml fresh culture medium containg 20% CS (v/v). Individual wells were assayed for cytotoxic activity against ^{51}Cr-labelled P815 on day 7. P(O) represents the proportion of non-responding wells for each dilution of responding cells.

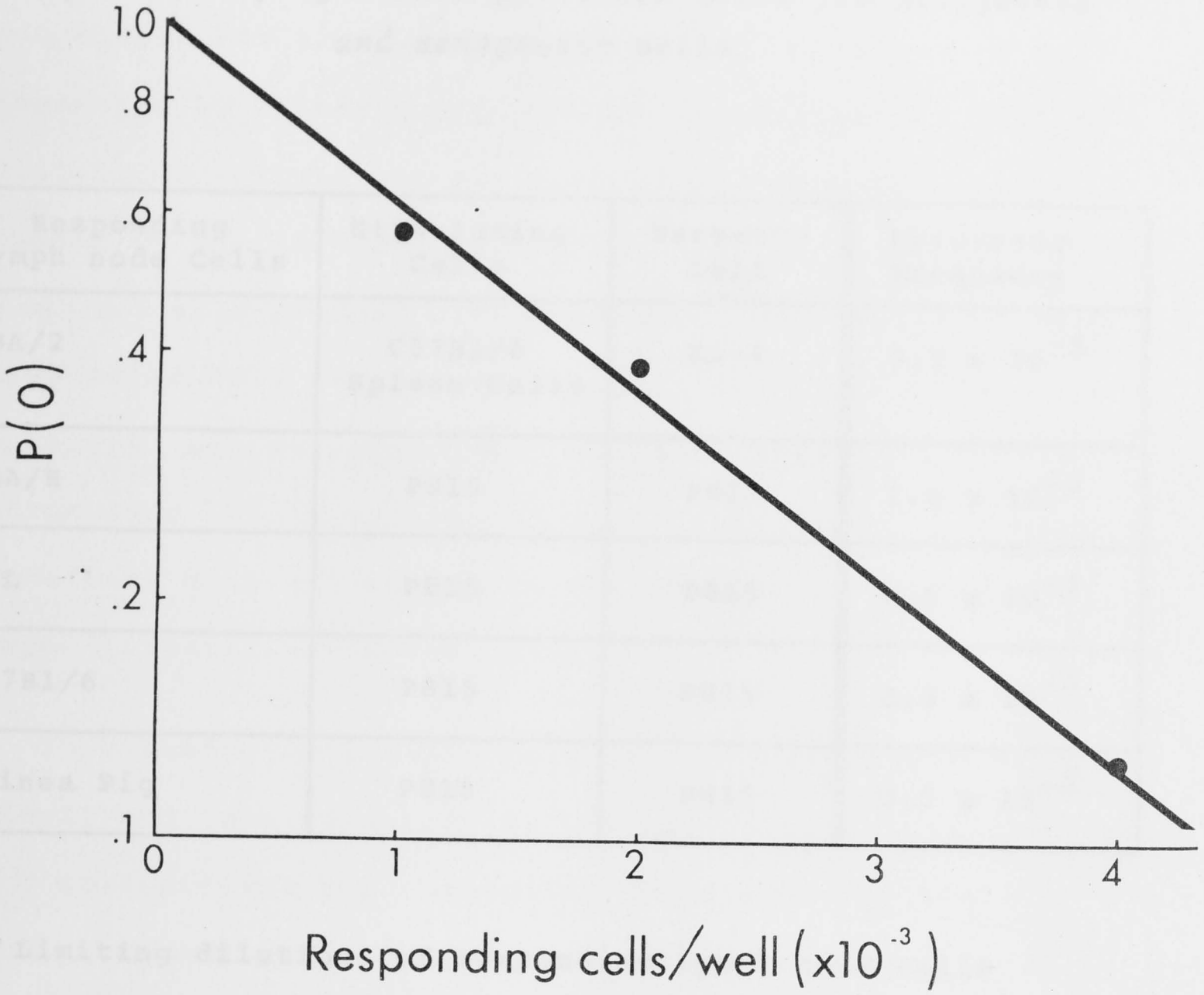

1.0
.8
.6
.4
.2
.1
P(O)
0
1
2
3
4
Responding cells/well (x10^-3)

TABLE 5.3

Frequencies of cytotoxic precursor cells for allogeneic and xenogeneic cells

Responding Lymph node Cells	Stimulating Cells	Target† Cell	Precursor Frequency
DBA/2	C57Bl/6 Spleen Cells	EL-4	0.5×10^{-3}
CBA/H	P815	P815	1.0×10^{-3}
SJL	P815	P815	0.5×10^{-3}
C57Bl/6	P815	P815	3.3×10^{-3}
Guinea Pig	P815	P815	0.5×10^{-3}

Limiting dilutions of responding lymph node cells were mixed with γ-irradiated stimulating cells in 96 well trays in CS containing medium as described in Section 2.10.

† Target cells were labelled with ^{51}Cr (Section 2.8).

leucocytes respond to stimulation by either goat or sheep cells but neither respond when cultured with rat cells. Similarly, rat leucocytes respond to stimulation by incompatible rat or mouse cells, but are unresponsive to stimulation by either sheep or goat leucocytes. A similar phylogenetic restriction was seen in the cytotoxic cell response to the P815 mouse tumour. In this case, both rat and mouse cells produce cytotoxic cells when cultured with γ-irradiated P815 whereas guinea pig, bovine or human lymphocytes are unresponsive. The same phylogenetic restrictions of immunological reactivity has been described in the case of graft versus host reactions in the chicken embryo (Lafferty and Jones, 1969).

In a theoretical analysis of allogeneic interactions Lafferty and Cunningham (1975) proposed a two signal mechanism for T cell activation. According to this model, the stimulating cell provides a source of foreign antigen in conjunction with an inductive stimulus - the lymphocyte costimulator - and both factors are required for lymphocyte activation (Section 4.4). It was proposed that costimulator production is dependent on the metabolic activity of the stimulating cell, and that the species specificity of alloreactivity is expressed at the level of this inductive signal.

Costimulator activity for cytotoxic T cell activation is present in the supernatant of con A activated leucocytes (CS) (Section 4.3.3). The data described in this chapter shows CS to be species specific. Both rat and mouse CS facilitate

the cytotoxic T cell response of mouse cells to UV-irradiated P815, while guinea pig CS is inactive. The guinea pig factor will, however, facilitate the response of guine pig cells to the mouse tumour, and this response is specific for the stimulating cell. This phylogenetic specificity of the second signal is essentially the same as that seen in the direct cellular interactions. The fact that costimulator is species specific indicates a hormone-like effect on the responsive lymphocyte and not simply some non-specific medium conditioning effect.

The failure of guinea pig cells to generate a cytotoxic cell response when cultured with the mouse tumour is not due to a lack of precursor cells for mouse antigens in the guinea pig cell population. Limit dilution analysis carried out in the presence of CS show the precursor frequency of guinea pig cells, for the P815 tumour, to be the same as that observed for mouse cells responding to alloantigens. Clearly, the phylogenetic restriction of leucocyte interactions operates at the level of the second signal.

In this light it is possible to account for some of the conflicting reports concerning the species specificity of mixed leucocyte reactivity. Lymphocyte populations have the capacity to recognize both allogeneic and xenogeneic histocompatibility antigens. However, antigen recognition alone is not sufficient for lymphocyte activation. The responsive cell is only triggered when costimulator activity, homologous with the responsive cell, is present in the culture systems. The activation of leucocytes by mitogens (Paetkau

et al., 1976; Section 4.3.3), by MLC stimulation (Altman and Cohen, 1975; Shaw *et al.*, 1978; Sopori *et al.*, 1977) and by the incubation of pre-primed cells with the primary antigen (Plate, 1976; Ryser *et al.*, 1978) result in the release of costimulator activity. Mitogenic effects, involving the addition of foetal calf serum and 2-ME to cultured leucocytes, have been reported (Lemke and Opitz, 1976; Goodman and Weigle, 1977). Thus, if the culture system is clean and the only source of costimulator is the stimulating cell population, reactivity will not be observed between phylogenetically unrelated cells. However, if the culture conditions result in the activation of the responsive cells to produce costimulator, responses to xenoantigens will be observed.

5.5 SUMMARY

In vitro responses to foreign histocompatibility antigens are phylogenetically restricted. Responses occur most readily to allogeneic or closely-related xenogeneic leucocytes, but not to unrelated xenogeneic cells. Specific cytotoxic responses to xenogeneic cells can be generated in the presence of a con A-stimulated leucocyte supernatant (CS) homologous with the responding cells. CS is also phylogenetically restricted in its action. Guinea pig lymphocyte populations contain a high frequency of xenoreactive cytotoxic cell precursors.

CHAPTER SIX

GENERATION OF HOMOGENEOUS POPULATIONS OF ALLOREACTIVE T CELLS *IN VITRO*

6.1 INTRODUCTION

Several groups have shown that specific T cell activation *in vitro* is a two signal process requiring the presentation of antigen to the responsive T cell in combination with an inductive factor (Bach *et al.*, 1977; Shaw *et al.*, 1978; Chapter 4). This factor we have called the lymphocyte costimulator (Section 4.4). Costimulator activity is present in the supernatant of con A-activated spleen cells (CS) Paetkau *et al.*, 1976; Chapter 4) and, at a lower level of activity, in the supernatant of mixed leucocyte culture (Altman and Cohen, 1975; Sopori *et al.*, 1977; Shaw *et al.*, 1978). This factor shows no strain specificity (Shaw *et al.*, 1978; Section 4.3.5) but has the same phylogenetic specificity as cellular alloreactivity (Chapter 5).

Removal of CS from the culture system early in the response aborts the development of cytotoxic cells suggesting that costimulator or another factor (maintenance activity) in the CS preparation is required for the continuous proliferation and differentiation of activated T cells in culture (Section 4.3.4). In this report we show that CS can be used to maintain the proliferation of alloreactive cells and so produce a homogeneous population of alloreactive T cells. In this respect our data confirms the report of Gillis and Smith (1977) that the subculture of antiviral cytotoxic T cells in the presence of CS results in the continued growth of these cells. However, in the case of alloreactive cells, we have been unable to maintain the continued growth of activated T cells in the presence of CS.

6.2 METHODS

6.2.1 *Generation and assay of cytotoxic cells.*

γ-irradiated P815 ($10^{5.2}$) or spleen cells ($10^{6.2}$) were cultured with responding lymph node cells (10^6) in 1 ml F-15 culture medium as described in Section 2.8. Cytotoxic activity was assayed against ^{51}Cr-labelled target cells as described in Section 2.8 and is expressed as cytotoxic units per culture (CU/culture) (Chapter 3).

Cultures were carried out in F-15 culture medium (Section 2.2) except where otherwise specified.

6.2.2 *Tritiated-thymidine (^{3}H-T) uptake*

Cells to be tested for ^{3}H-T uptake were suspended in culture medium at a viable cell density of 2.5×10^5/ml and were added to flat-bottomed 96 well culture trays (Linbro, IS-FB-96-TC) in quadruplicate, 0.2 ml volumes. To each well was added 0.025 ml of ^{3}H-T (150 µCi/ml). Thymidine-uptake was estimated 5 hours later as described in Section 2.7.

6.2.3 *Preparation of con A-activated cell supernatants (CS)*

CS was prepared from mouse (CBA/H) and guinea pig spleen cells as described in Section 2.9. The guinea pig CS preparation was concentrated 40-fold.

6.2.4 *Treatment with anti-Thy 1.2 serum.*

This was carried out as described in Section 2.11.

6.2.5 *Rosetting for Ig^+ cells.*

This was carried out as described by Parish *et al* (1974). Sheep erythrocytes were coated, via $CrCl_3$, with sheep anti-mouse Ig and were mixed with the cultured cell suspensions at 4°C. The suspensions were then stained with crystal violet (2% v/v) and rosettes counted in a haemocytometer immediately after staining.

6.2.6 *Immunoperoxidase staining of surface and intracellular Ig.*

The horseradish peroxidase (HPO) labelled sheep anti-rabbit Fab reagent was prepared exactly as described for HPO-sheep anti-rat Fab by Nawa *et al* (1978). Other techniques were also carried out essentially as described (Nawa *et al.*, 1978).

Samples of cell suspensions (0.2 ml at 2×10^6/ml) were cytocentrifuged, dried, and fixed for 15 mins with 4% paraformaldehyde. The slides were washed three times in Tris-buffered saline (TBS) and incubated with rabbit anti-mouse Ig (Dakopatts, Z109) at 1/5 and 1/10 dilutions in TBS in a moist chamber at room temperature for 2 1/2 hrs. This reagent was centrifuged at 17,000 g for 1 hr prior to use to remove aggregates. The slides were then washed 3 times in TBS and incubated with the HPO-sheep and anti-rabbit Fab (1/20) for 45 mins at room temperature. The slides were washed three times in Phosphate-buffered saline and stained for peroxidase for 5 mins with 3-amino-9-ethylcarbazol and hydrogen peroxide (Graham *et al.*, 1965). This technique stains both intracellular and surface Ig (Stanislawski *et al.*, 1976).

6.2.7 *Limiting dilution assay.*

This was carried out as described in Section 2.10.

6.2.8 *Histology.*

Preparations for light microscopy were produced by centrifuging 0.5 ml volumes of 1% bovine serum albumin (SIGMA, BSA) in Hank's BSS containing 1-2 x 10^5 cells onto glass slides using a Shandon Elliott-cytospin cytocentrifuge (1400 rpm for 5 minutes). These cells were stained with Leishman's stain.

6.3 RESULTS

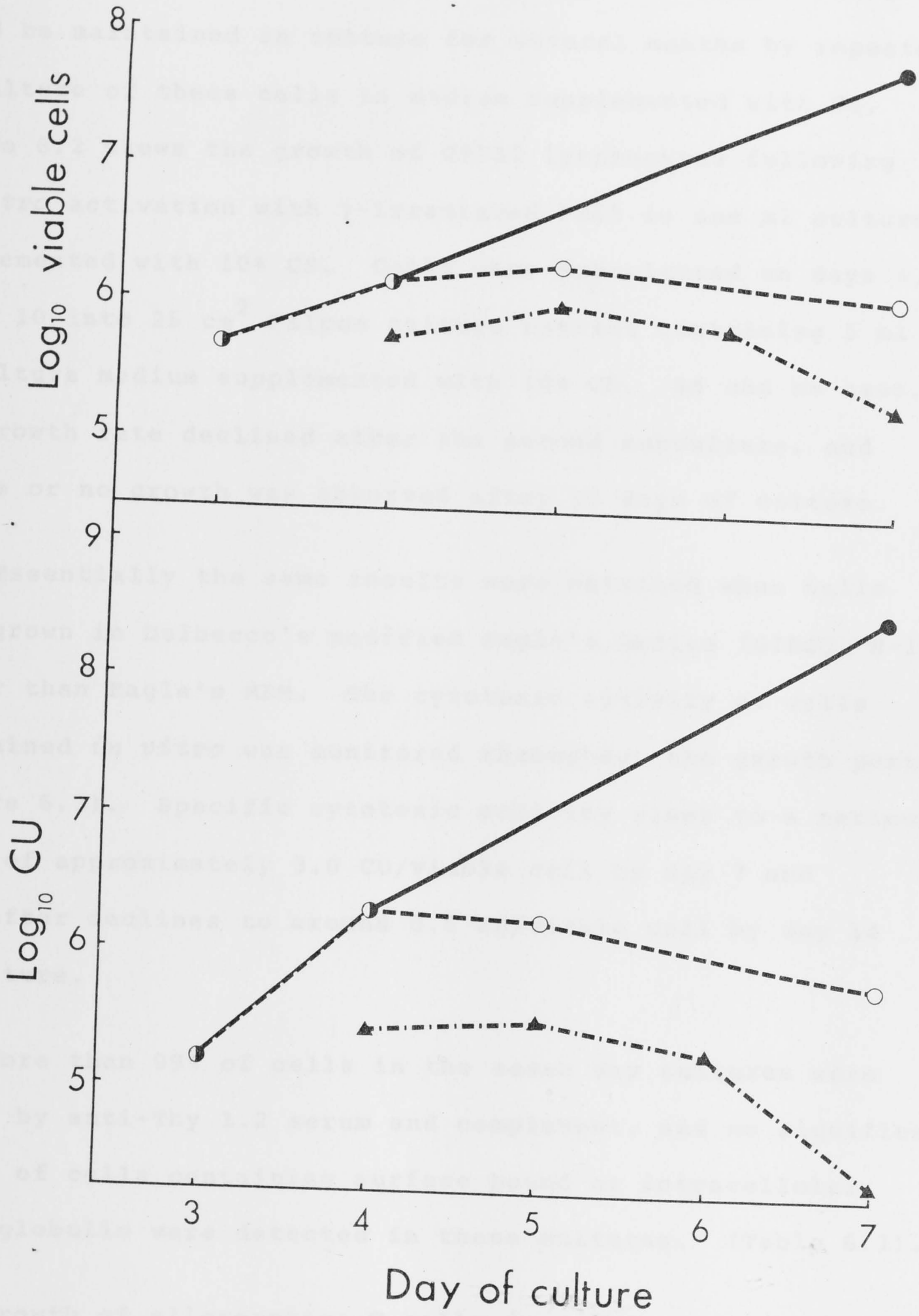
6.3.1 *Characteristics of cytotoxic T cell growth.*

Primary cytotoxic T cell responses to alloantigen can be generated *in vitro* using a variety of γ-irradiated stimulator cells (Section 4.3.1). These responses peak on the 5th day of culture and subsequently decline (Section 4.3.1). Such responses can be enhanced by the addition of a con A-stimulated spleen cell supernatant to the culture medium. Figure 6.1 shows the response of C57Bl lymph node cells to γ-irradiated P815 under different culture conditions. The addition of CS (10% v/v) to 1 ml cultures significantly amplified the response, but there was still a loss of cytotoxic activity after day 5 of culture (Fig. 6.1B). However, when 1 ml cultures were harvested on day 4 and added to 75 cm^2 Falcon flasks containing 30 ml of fresh F-15 culture medium supplemented with 10% CS, continued exponential growth of cells and cytotoxic activity was maintained through to the 7th day of culture (Figure 6.1).

Figure 6.1 Response of C57Bl/6 lymph node cells to γ-irradiated P815. One ml cultures were set up without added CS (▲-·-·-▲), in medium containing 10% CS (O- - -O), or in medium containing 10% CS and subcultured on day 4 into 75 cm^2 flasks containing 30 ml culture medium and 10% CS (●———●).

A. Total viable cells/culture,

B. Total cytotoxic units (CU)/culture.


Log10 viable cells
8
7
6
5
9
8
7
6
5
Log10 CU
3
4
5
6
7
Day of culture

Gillis and Smith, (1977) have reported that murine cytotoxic T cells specific for virus-infected target cells could be maintained in culture for several months by repeated subculture of these cells in medium supplemented with CS. Figure 6.2 shows the growth of C57Bl lymphocytes following *in vitro* activation with γ-irradiated P815 in one ml cultures supplemented with 10% CS. Cells were subcultured on days 4, 7 and 10 into 25 cm^2 Falcon culture bottles containing 5 ml of culture medium supplemented with 10% CS. As can be seen, the growth rate declined after the second subculture, and little or no growth was observed after 10 days of culture.

Essentially the same results were obtained when cells were grown in Dulbecco's modified Eagle's medium (GIBCO, H-16) rather than Eagle's MEM. The cytotoxic activity of cells maintained *in vitro* was monitored throughout the growth period (Figure 6.3). Specific cytotoxic activity rises to a maximum value of approximately 3.0 CU/viable cell by day 7 and thereafter declines to around 0.5 CU/viable cell by day 14 of culture.

More than 99% of cells in the seven day cultures were killed by anti-Thy 1.2 serum and complement, and no significant number of cells containing surface bound or intracellular immunoglobulin were detected in these cultures. (Table 6.1).

Growth of alloreactive T cells *in vitro* requires the continuous presence of CS in the culture medium Cells washed free of CS on the 4th or 7th day of culture rapidly lose their ability to take up tritiated-thymidine and their cytotoxic activity (Table 6.2).

Figure 6.2 Viable cell numbers in cultures of γ-irradiated P815 and C57Bl/6 lymph node cells maintained in medium supplemented with 10% CS. One ml cultures, set up on day 0, were subcultured on day 4 at a cell density of $2x10^4$ viable cells/ml, into 25 cm^2 flasks containing 5 ml of culture medium. Further subcultures on days 7 and 10 were made in 25 cm^2 flasks at a cell density of 10^5 viable cells/ml.

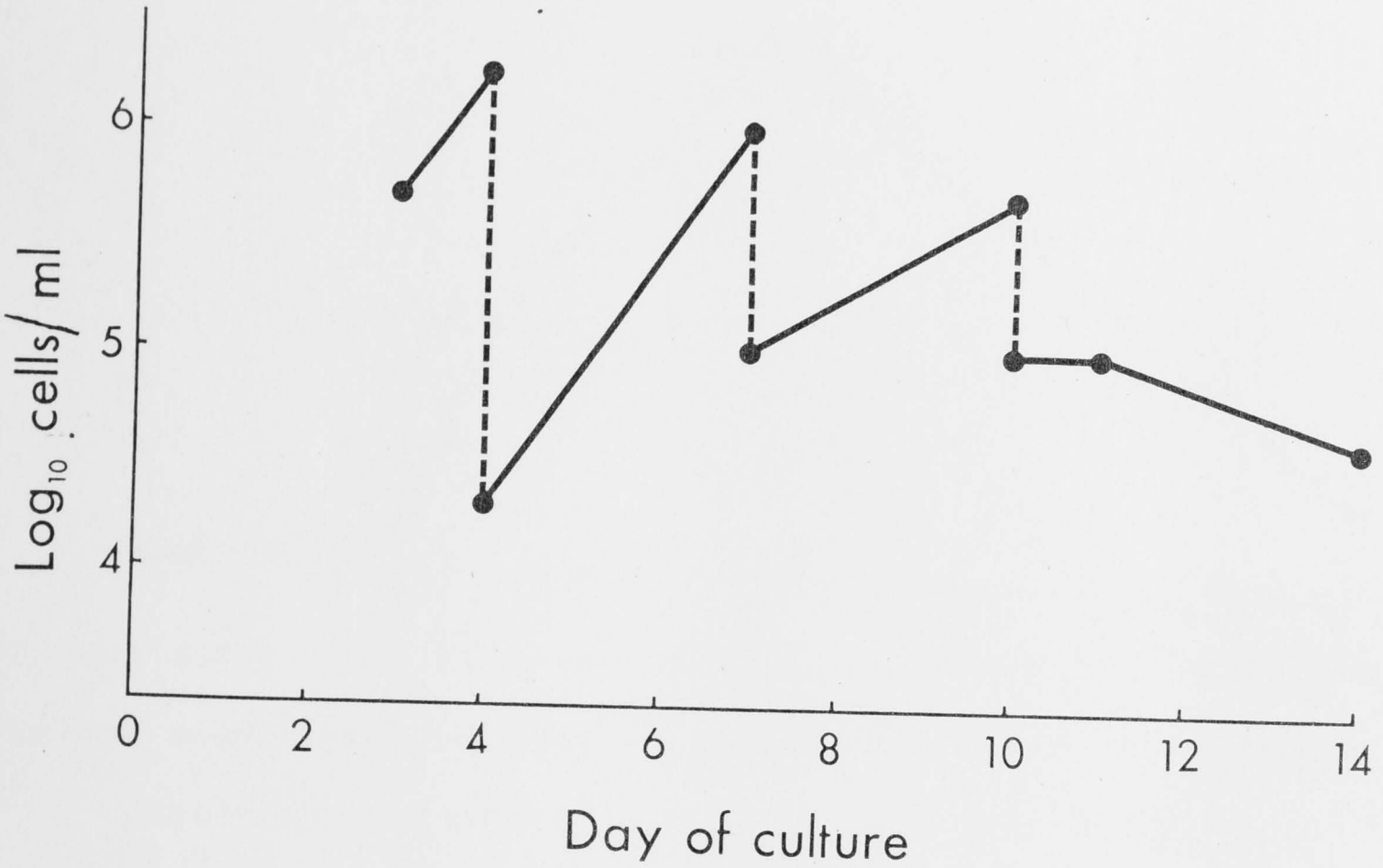

Log10 cells/ml
6
5
4
0
2
4
6
8
10
12
14
Day of culture

Figure 6.3 Cytotoxic activity of cells taken from long term cultures. Cells were harvested at various times from cultures set up as described (Fig. 6.2) and tested for cytotoxic activity against ^{51}Cr-labelled P815.

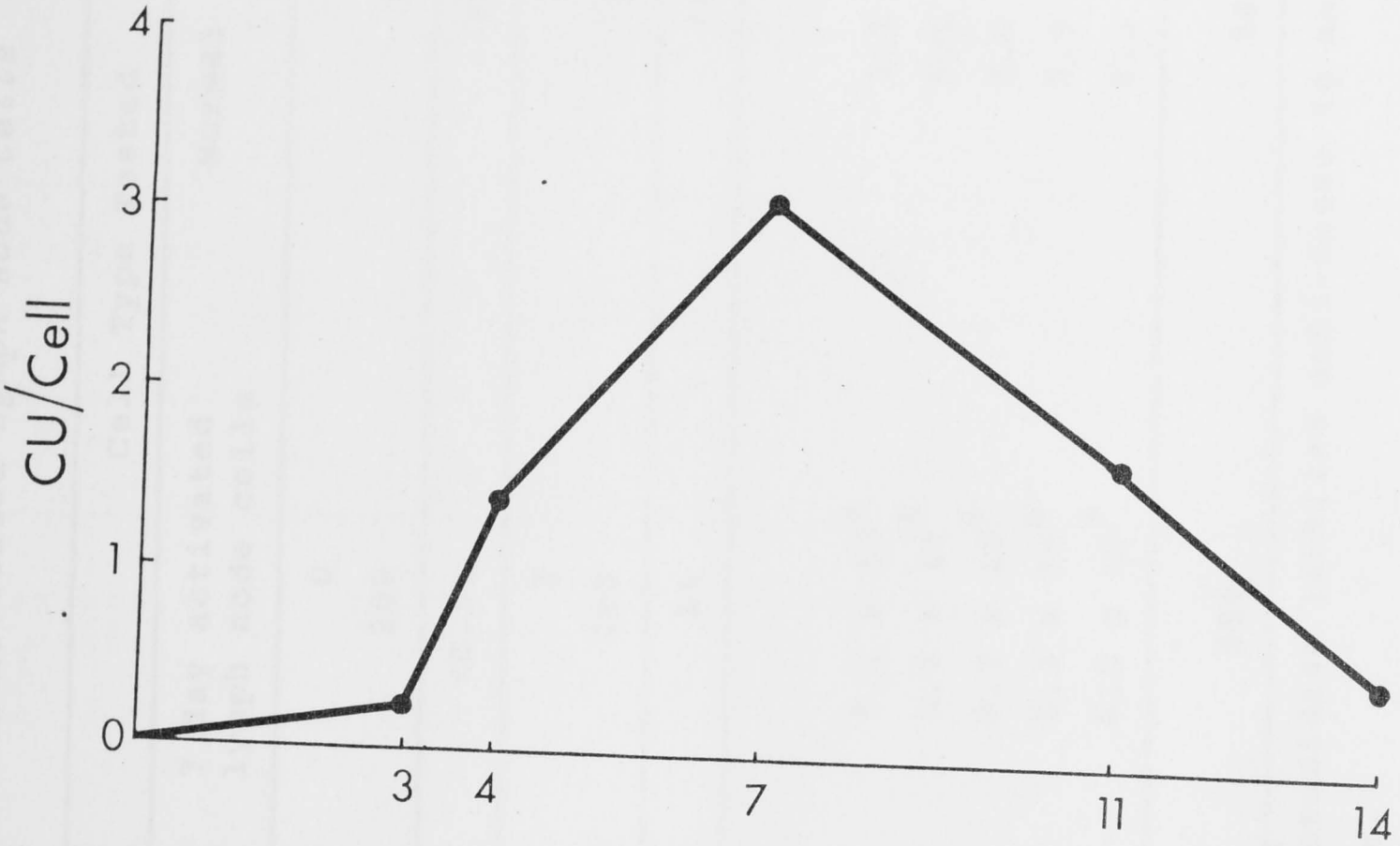

CU/Cell
4
3
2
1
0
3
4
7
11
14
Day of culture

TABLE 6.1

T and B Cell Composition of Normal and Activated Lymph Node Cells

TREATMENT		Cell Type Tested	
		7 day activated lymph node cells	Normal Lymph node cells
Surface Ig Staining[1]	+ve	0	38
	-ve	200	62
% Surface Ig +ve cells		<0.5%	38%
EA-Rosette Formation[2]	+ve	2	42
	-ve	198	58
% EA-Rosette Positive Cells		1%	42%
Viable cells after treatment of 6×10^5 cells with:			
Normal Ascitic Fluid + Complement		5.2×10^5	5.2×10^5
a-Thy 1.2 Ascitic Fluid ($\frac{1}{2}$) + --		5.2×10^5	Not tested
a-Thy 1.2 Ascitic Fluid ($\frac{1}{16}$)+ Complement		8.7×10^4	2.0×10^5
a-Thy 1.2 Ascitic Fluid ($\frac{1}{8}$) + Complement		5.3×10^4	2.9×10^5
a-Thy 1.2 Ascitic Fluid ($\frac{1}{2}$) + Complement		6.0×10^3	2.3×10^5
% Cells Killed by anti-Thy 1.2 antibody and complement:		99%	54%

1 Staining for surface or intracellular Ig with peroxidase labelled anti-mouse Ig antibody.

2 Rosetting with anti-mouse Ig coated erythrocytes.

TABLE 6.2

Cytotoxic and Proliferative Activity of Activated T cells cultured for 2 days in the presence or absence of CS

Cells Tested	CS[1] Added	^{3}H-Thymidine[2] Uptake	Cytotoxic Units/Cell
4 day activated cells	+	58,212	3.4
	-	89	<0.1
7 day activated cells	+	50,000	4.6
	-	311	0.2

1 CS added at a concentration of 10% (v/v)

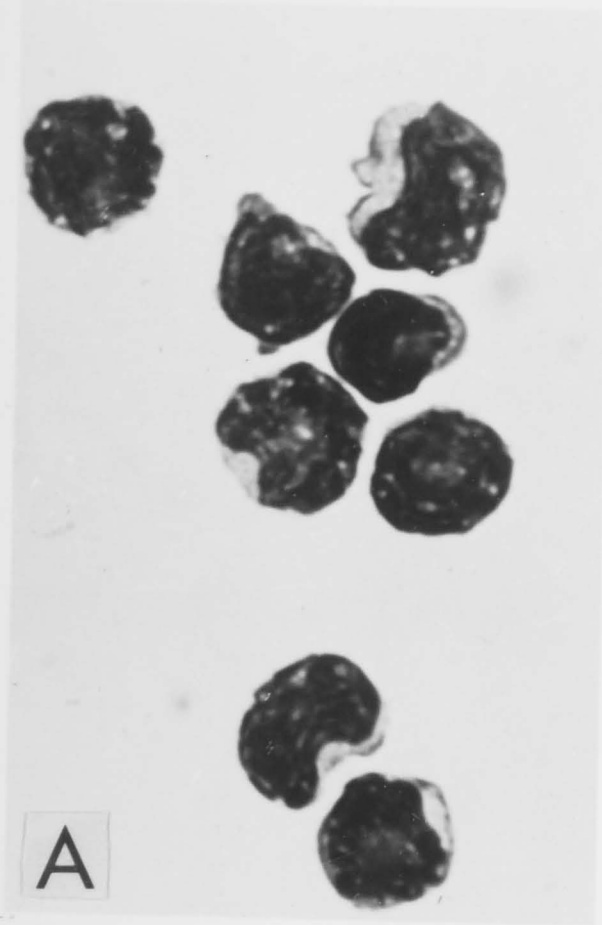
2 CPM/10^{6} viable cells

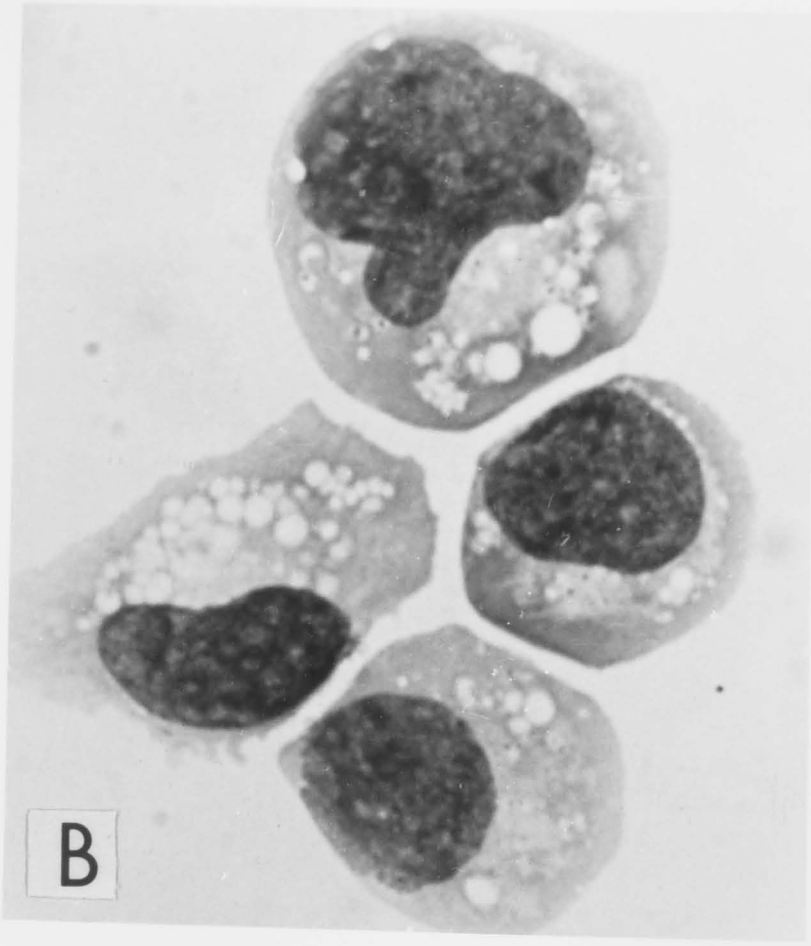
The morphology of the cultured cells was examined at various times over a 14 day period. γ-irradiated P815 tumour cells were mixed with C57Bl lymph node cells in medium supplemented with 10% CS. The cells were subcultured on days 4, 7 and 10 into 75 cm^2 Falcon flasks (Falcon, 3024) containing 30 ml culture medium and 10% CS, at cell densities of 3×10^4/ml on day 4 and 10^5/ml on days 7 and 10. A similar growth curve as described above (Fig. 6.2) was observed using this technique.

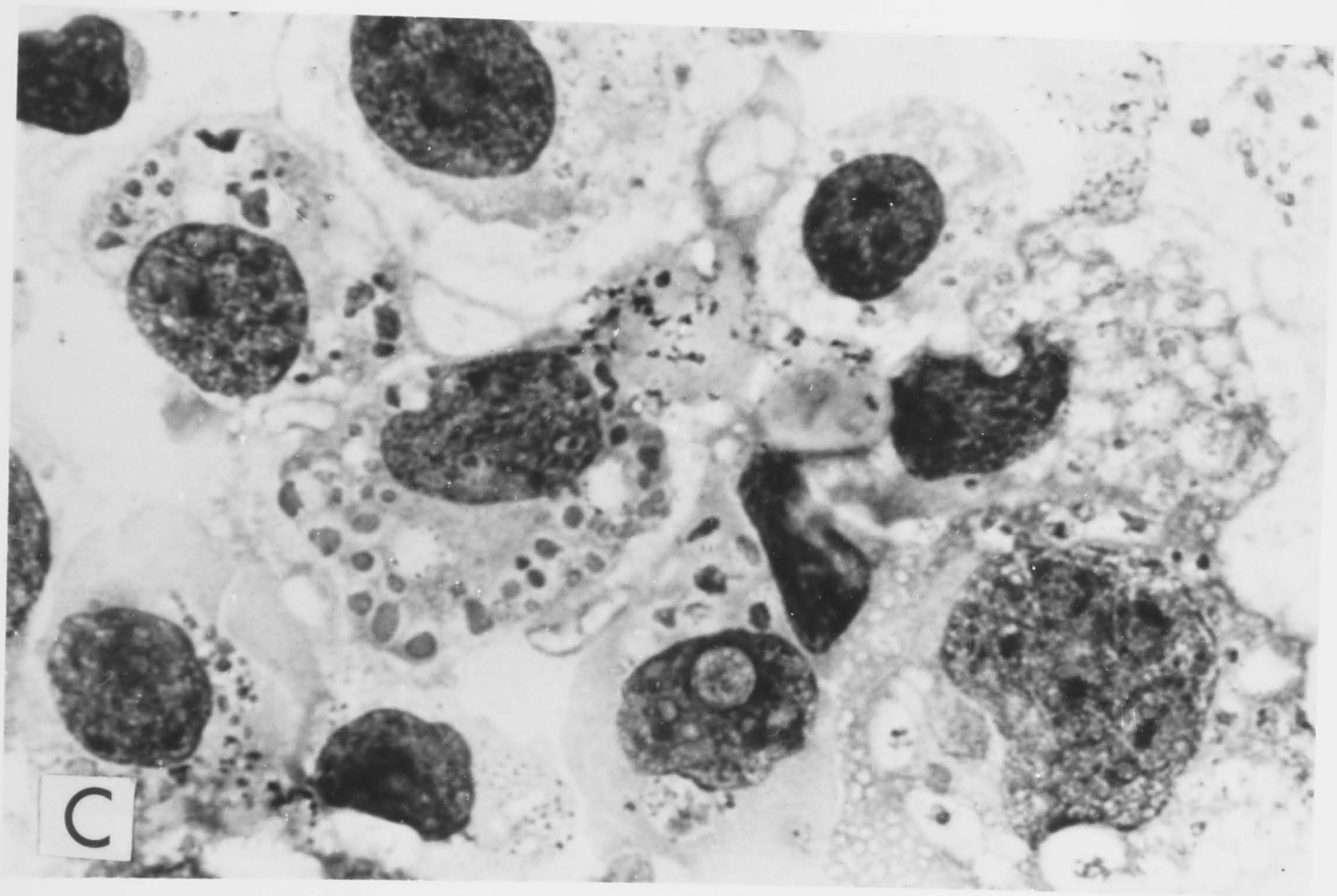
Normal C57Bl lymph node cells, day 7 and day 14 cultured cells were taken for histological examination. Figure 6.4 shows Leishman stained smears of these three cell populations. By day 7 the cultures consist of a homogeneous population of activated lymphocytes (Fig. 6.4B) in contrast to the predominantly small lymphocyte population at day 0 (Fig. 6.4A). Day 7 cultures consistently contained at least 95% blast cells with fewer than 10% contaminating dead cells as assayed by trypan blue uptake. By 14 days, cultures showed signs of cellular degeneration (Fig. 6.4C). When examined with the electron microscope, some cells showed frank cytoplasmic disorganisation and approximately 50% of cells in these cultures had large cytoplasmic vacuoles containing myelin figures and other membranous debris. Thus, CS can be used to maintain the growth of activated T cells *in vitro*. After seven days, cultures consist of a homogeneous population of activated T cells with a high level of specific cytotoxic activity. Further subculture in the presence of CS results in a decline in cellular growth rate, a fall in cytotoxic activity and the gradual degeneration of the T cell cultures. In the case

Figure 6.4 · Morphologic changes in cultures of C57Bl/6 lymphocytes activated *in vitro* by γ-irradiated P815.

Lymph node cells stained on A, day O, B, day 7, and C, day 14. Leishman's stain. X 1250.


A


B


C

of alloreactive T cells, CS cannot be used to maintain the indefinite growth of cytotoxic T cell cultures.

6.3.2 *Clonal analysis of the activated T cell population.*

The following experiments were designed to determine the clonal origin of the cell population giving rise to the homogeneous T cell cultures. Activated T cells were generated by mixing γ-irradiated P815 and C57Bl lymph node cells in standard 1 ml cultures without CS. On the fourth day of culture, the responding cells were harvested and cloned in the presence of γ-irradiated P815, using the limit dilution assay described in Section 2.10. Figure 6.5 shows a comparison of limit dilution assays carried out on normal lymph node cells and the activated cells harvested from the four day cultures. During the four day culture period, there was a selective growth of alloreactive T cells with the precursor frequency of the cultured cells being 1:20 as compared with a value of 1:300 for the normal lymph node population.

Four day cultured cells were also set up in a limit dilution assay at a cell density of 10 cells/well i.e., an average of 0.5 cytotoxic precursors/well. After a further six days of culture, each well was examined in two ways. First, each well in the assay tray was examined with an inverted microscope and those containing visible clones (50 or more blast cells) were scored. ^{51}Cr-labelled P815 cells were then added to each cup and the position of cytotoxic clones was determined by assaying ^{51}Cr-release. Table 6.3 shows the results of this experiment. Most wells with visible clones (25/27) were cytotoxic, and of the cups that lacked visible

Figure 6.5 Cytotoxic responses of normal C57Bl/6 lymph node cells (■——■) and C57Bl/6 lymph node cells after 4 days of culture with γ-irradiated P815 (▲-·-·-▲), to ^{51}Cr-labelled P815 in a limit dilution assay. P(O) represents the proportion of non-responsive wells for each given dilution.

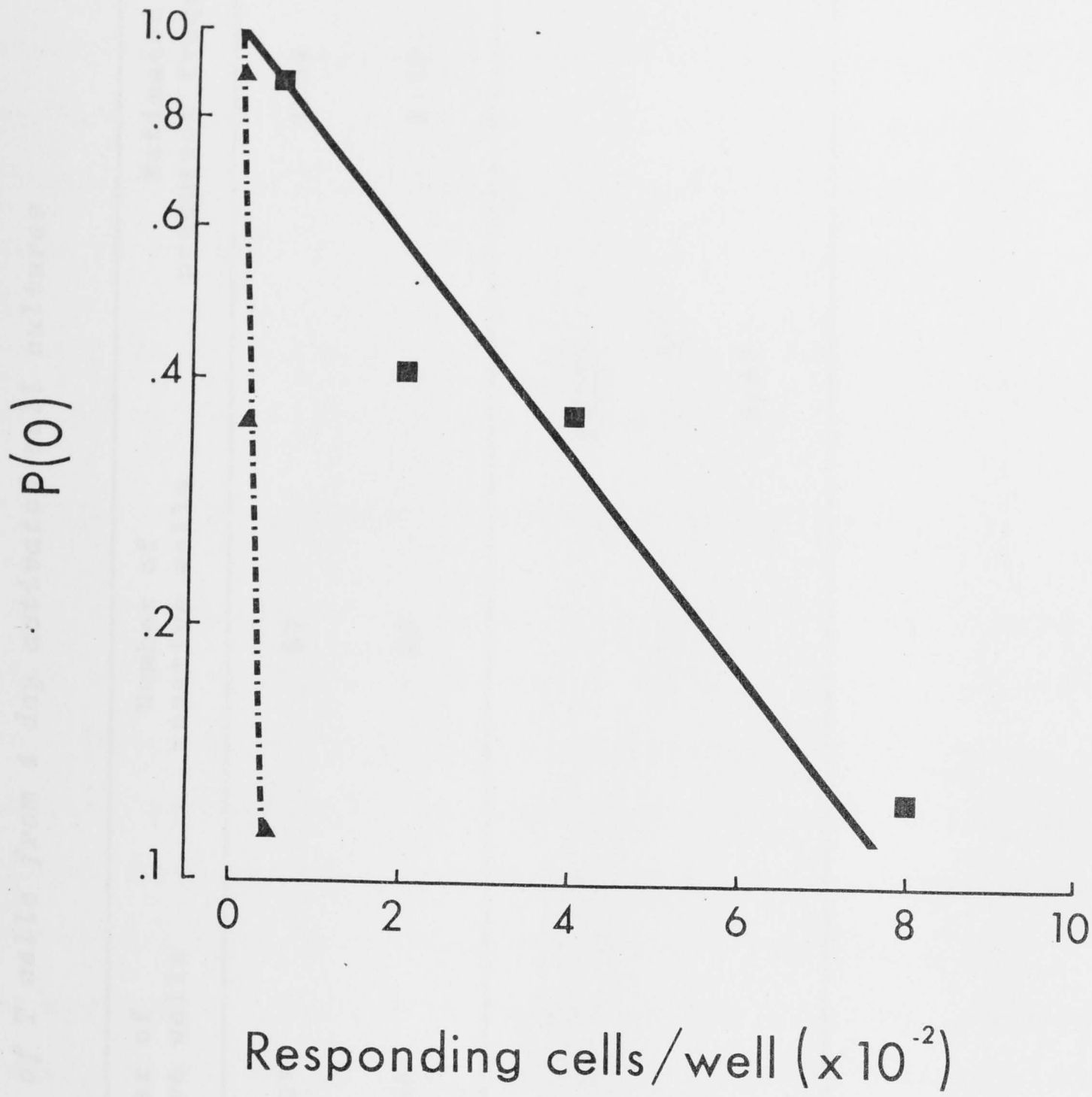

P(O)
1.0
.8
.6
.4
.2
.1
0
2
4
6
8
10
Responding cells/well (x 10^-2)

TABLE 6.3

Clonal analysis of T cells from 4 day activated cell cultures

Parameter examined	Number of positive wells	Number of negative wells	Estimated precursor frequency
Presence of cellular proliferation	27	67	1:29
Presence of cytotoxic activity	34	60	1:22
Fraction of blast cell +ve wells that contain cytotoxic activity		25/27	
Fraction of blast cell -ve wells that contain cytotoxic activity		9/67	

clones few (9/67) showed cytotoxic activity. In these wells very low levels of cytotoxic activity (<3.8% lysis) were observed; cytotoxic activity in positive wells ranged from 1.8 to 85% lysis. Also, the two wells containing visible blast cells but demonstrating no detectable cytotoxic activity appeared to be very small clones (<100 blast cells/well).

The precursor frequency of cytotoxic (1:22) and proliferative (1:29) clones was approximately the same.

6.3.3 *Species specificity of the maintenance factor.*

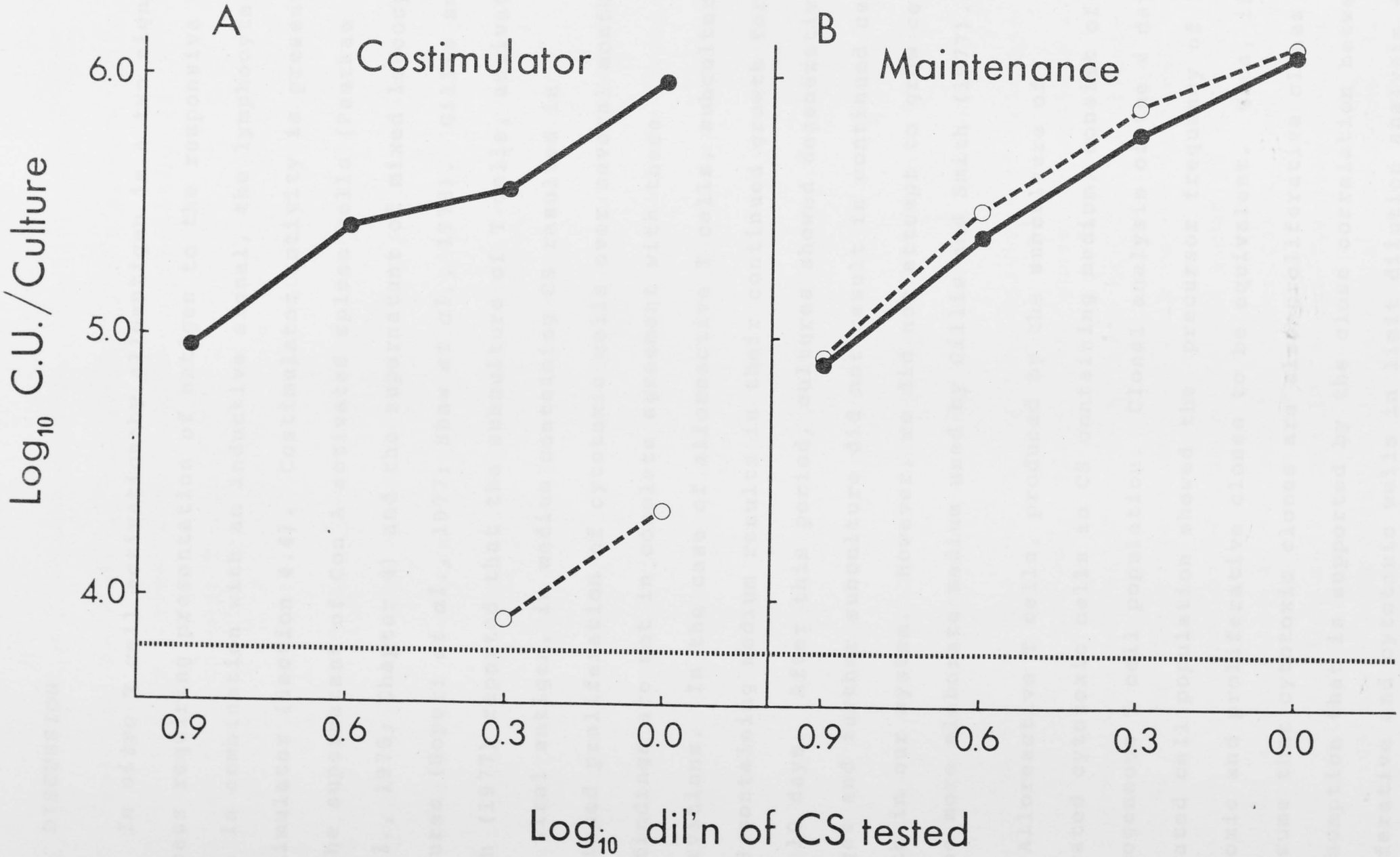
Our studies of costimulator activity in CS preparations (Chapter 5) showed this factor to be species specific in its effect. CS of guinea pig origin was inactive for mouse lymphocytes responding to UV-irradiated allogeneic cells, but was active for responding guinea pig lymphocytes. Similarly, mouse CS had little or no activity in cultures containing guinea pig lymphocytes but was quite potent in mouse lymphocyte cultures. Gillis *et al* (1978) reported that the T cell growth factor required for the continuous growth of T cells in culture was not species specific. It is possible therefore, that the costimulator and maintenance effects of CS preparations may be due to different activities.

In the following experiment, we compared the relative activities of one batch of mouse and guinea pig CS in both costimulator and maintenance assays. Maintenance activity of the CS preparations was assayed by measuring the capacity of dilutions of CS to maintain the development of cytotoxic activity in cultures containing cells harvested four days after primary

activation. Activated cells were obtained by mixing γ-irradiated P815 ($10^{5.2}$cells/culture) and C57Bl lymph node cells (10^6 cells/culture) in one ml cultures supplemented with 0.1 ml of mouse CS. On day 4 cells were harvested, washed, and resuspended at 10^5 viable cells/ml in fresh culture medium without CS. One ml volumes of this cell suspension were then added to culture plates containing 0.1 ml volumes of serial 2-fold dilutions of either guinea pig or mouse CS. These cultures were incubated for a further 3 days at 37°C and assayed for cytotoxic activity. The results of this study (Figure 6.6B) show that these mouse and guinea pig CS preparations have essentially the same maintenance activity.

Costimulator activity of the same CS preparations was then assayed by measuring the capacity of dilutions of CS to facilitate the activation of cytotoxic activity in cultures of UV-irradiated P815 (S^-) and C57Bl lymph node cells (Section 4.3.3). Figure 6.6A shows a comparison of the costimulator activity of the mouse and guinea pig CS using C57Bl lymphocytes as responding cells. It can be seen that the guinea pig CS has little activity, compared to mouse CS, in a system containing mouse responding cells. Thus maintenance and costimulator activities have differing species specificities and are therefore separate activities. The preparation of guinea pig CS used in these experiments was shown, in a separate experiment, to have costimulator activity for guinea pig cells (Section 5.3.2).

Figure 6.6 Assay of mouse and guinea pig CS preparations for costimulator and maintenance activity using mouse responsive cells. Mouse CS (●———●). Guinea pig CS (O---O).


A
Costimulator
B
Maintenance
6.0
5.0
4.0
Log_{10} C.U./Culture
0.9
0.6
0.3
0.0
0.9
0.6
0.3
0.0
Log_{10} dil'n of CS tested

6.4 DISCUSSION

In vitro T cell activation by alloantigen is a two signal process requiring presentation of antigen to the responsive T cell in combination with an inductive signal, the lymphocyte costimulator (Section 4.4). Costimulator activity is present in the supernatant of Con A activated spleen cells (Paetkau *et al.*, 1976; Chapter 4) and the supernatant of mixed leucocyte cultures (Sopori *et al.*, 1977; Shaw *et al.*, 1978). Gillis and Smith (1977) reported that the subculture of T cells, activated to a viral antigen, in medium containing CS resulted in continued proliferation of cytotoxic cells over several months. Our findings are not in complete agreement with these observations. In the case of alloreactive T cells, subculture in CS containing medium results in their continued growth for 7 to 10 days. After this period, cultures showed degenerative changes and further subculture did not result in continued cell growth in our system. However, we did not attempt to grow cells in the more elaborate medium used by Gillis and Smith (1977).

Alloreactive T cells, produced by the subculture of activated cytotoxic cells in CS containing medium, consist of a homogeneous T cell population. Clonal analysis of the 4 day activated cell population showed the precursor frequency of cytotoxic and proliferative clones to be equivalent. Thus, if we assume that cytotoxic clones are also proliferative clones - an assumption that is supported by the close correlation between proliferative and cytotoxic wells in limit dilution analysis - then the 4 day activated T cell population produced in response to the P815 tumour did not contain detectable numbers of cells

with proliferative but not cytotoxic potential. Thus the cell population produced by subculture of activated T cells in CS containing medium is a homogeneous population of alloreactive cytotoxic T cells.

Costimulator activity of CS preparations is required early in the process of T cell activation (Section 4.3.4), and the continued expression of cytotoxic activity by activated T cell populations also requires the presence of CS in the culture medium (Table 6.2). When CS is removed from cultures of activated T cells, these cells rapidly revert to a non-proliferating non-cytotoxic state. This late activity of CS preparations we refer to as maintenance activity. In Chapter 4 I suggested two possible explanations for this phenomenon (Section 4.4). The first was that CS contains only one activity (costimulator) that acts by interfering with an intrinsic "switch off" mechanism operating within the lymphocyte. According to this model, costimulator would be required early in the process of lymphocyte activation and its continued presence in the culture medium would be required to maintain these cells in the activated state. The other alternative was that costimulator and maintenance effects are due to different activities in the CS preparation. In this report we have shown that a preparation of guinea pig and mouse CS that have equivalent maintenance activities for mouse cells, have markedly different costimulator activities for mouse lymphocytes; the guinea pig CS has little costimulator activity for mouse cells although it is active for guinea pig cells (Section 5.3.2). Thus maintenance and costimulator activities have different species specificity and are therefore separable activities.

At this stage we do not know whether these two activities represent the function of two molecular species or whether they represent two activities of one bifunctional molecule. The finding that maintenance activity is not species specific is consistent with the report of Gillis and Smith (1977) that both human and mouse CS preparations would maintain the growth of virus specific cytotoxic T cells.

6.5 SUMMARY

The supernatant from Con A activated spleen cells (CS) can be used to generate homogeneous populations of alloreactive T cells *in vitro*. Subculture of activated cells in CS containing medium is required for this continued proliferation and expression of effector activity. Prolonged subculture in CS containing medium does not result in indefinite growth and proliferation of alloreactive T cells. The activity in CS required to maintain cytotoxic cell growth is not species specific, and is therefore separable from the costimulator activity in CS required for the initiation of the T cell response to alloantigen; this latter activity is species specific.

CHAPTER SEVEN

T CELL RESPONSES TO H-2I REGION CONTROLLED DETERMINANTS

7.1 INTRODUCTION

It has been found that only allogeneic viable cells of the reticulo-endothelial system have the capacity to stimulate T cell responses in mixed cell culture. Cells derived from other tissues, and lymphoid cells which have been metabolically inactivated by any of a variety of techniques have generally been found to be non-stimulatory (S^-) in mixed lymphocyte culture (MLC) (Section 1.4). A number of models have been proposed to account for these phenomena.

We have proposed a two signal mechanism of T cell activation (Section 4.4; Lafferty and Cunningham, 1975). Antigen (signal 1) as well as an inductive second signal (costimulator) is required for the activation of cytotoxic T cell responses. Both signals are normally provided by the S^+ stimulating cell, and metabolic inhibition of this cell destroys its capacity to produce signal 2 and therefore renders it non-stimulatory (S^-). Cells which intrinsically lack the capacity to produce signal 2 are also non-stimulatory.

Bach and co-workers (Bach, 1973; Bach *et al.*, 1976, 1977) have also proposed a two signal mechanism for T cell activation (Section 1.5). This model postulates that a distinct class of non-cytotoxic helper T cells are required for the activation of cytotoxic T cells *in vitro*. Helper T cells respond to antigenic determinants (LD determinants), which are coded by the I-region of the H-2 complex. Activated helper T cells then provide an inductive signal (signal 2) to the cytotoxic T cell precursors, either directly or via soluble factors.

In this chapter I present evidence that stimulation through I-region controlled determinants, like stimulation through H-2K/D controlled histocompatability antigens, generates alloreactive T cells with cytotoxic potential. We could find no evidence for the presence of a distinct class of non-cytotoxic helper T cells.

7.2 METHODS

7.2.1 *Animals.*

Male mice of the BALB/c ($H\text{-}2^d$), CBA/H($H\text{-}2^k$), C57Bl/6 ($H\text{-}2^b$), DBA/2 ($H\text{-}2^d$), A.TL (skkkd) and A.TH (ssssd) strains were used at 8-12 weeks of age (Section 2.1).

7.2.2 *Culture conditions and techniques.*

Mixed cell cultures were carried out essentially as described (Section 2.8). γ-irradiated stimulating spleen cells ($10^{6.2}$) were mixed with responding lymph node cells (10^6) in 1 ml F-15 culture medium in Multiwell tissue culture plates (Falcon, 3008). Cytotoxic activity was assayed against ^{51}Cr-labelled P815 target cells (Section 2.8) in the presence of concanavalin A (SIGMA, C-2010; 10 μg/ml), and is expressed as percent lysis which equals $\frac{\text{test release - control release}}{\text{water lysis release - control release}}$ x 100.

7.2.3 *Limiting dilution assay*

This was carried out as described (Section 2.10) except that cytotoxic activity was tested using ^{51}Cr-labelled P815 in the presence of Con A (10 μg/ml) as described above (Section 7.2.2). Individual wells were also examined visually for activated lymphocytes as described in Section 2.10. γ-irradiated

spleen cell stimulators ($10^{6.2}$/ml) were used in all cultures.

7.2.4 *Treatment with anti-Thy 1.2 and complement.*

This was carried out as described in Section 2.11.

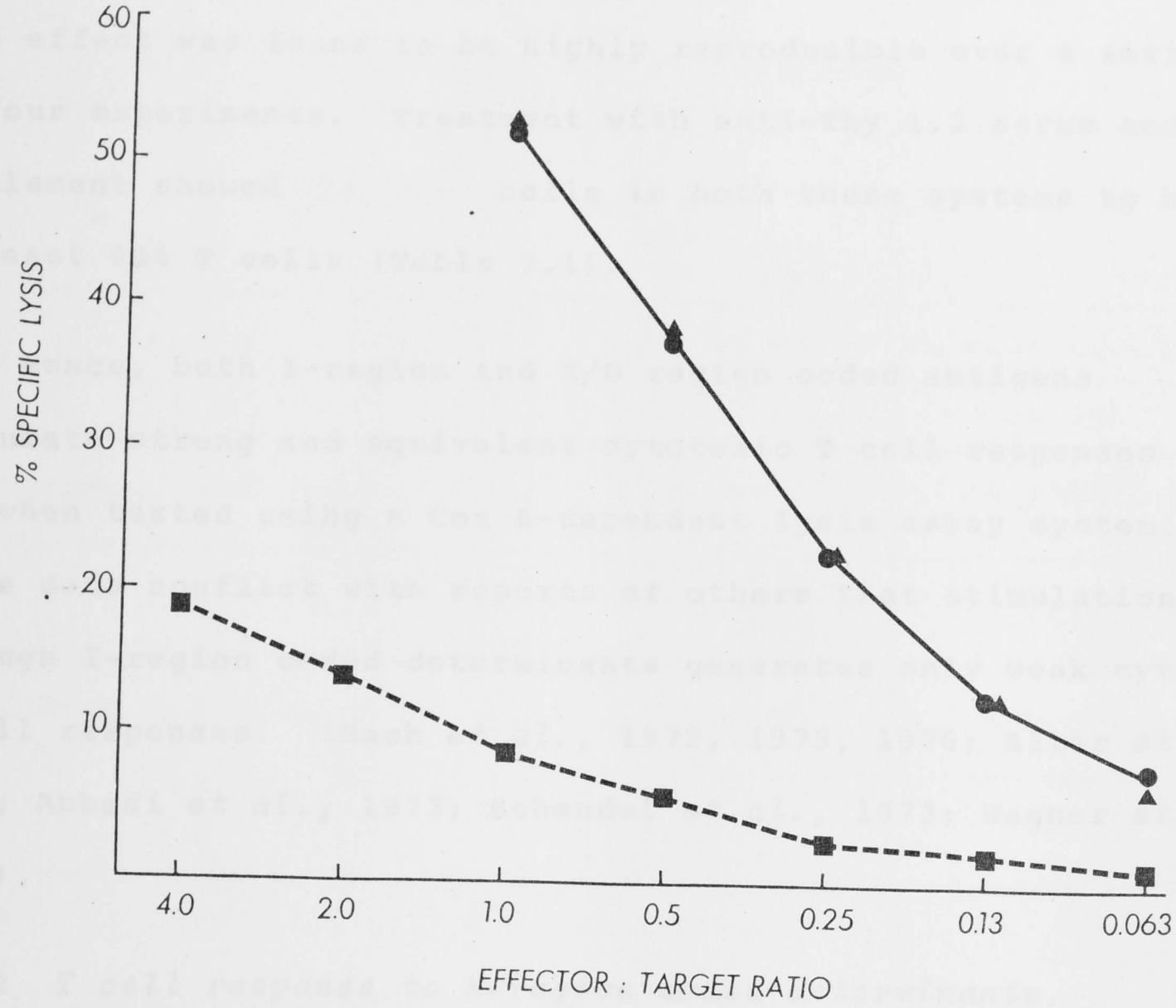
7.3 RESULTS

7.3.1 *T cell responses to H-2I and H-2K/D coded antigens.*

The Con A-dependent lysis of P815 tumour cells in a ^{51}Cr-release assay has been used as a non-specific assay for the detection of cytotoxic T cells activated in culture (Bevan and Cohn, 1975). This assay system has the advantage that the same target system can be used to compare cell populations activated to different antigens, thus avoiding variability resulting from differences in target cell lysability. We have used this system to test the Bach model (Bach *et al.*, 1976, 1977), which postulates that stimulation through H-2I coded determinants generates, predominantly, a non-cytotoxic helper T cell (T_H) response while stimulation through H-2K/D antigens generates, predominantly, a cytotoxic T cell (T_C) response.

MLC responses directed against either H-2I^s or H-2K/D^k controlled determinants alone were generated by mixing A.TL responding lymph node cells with either A.TH (I-region different) or CBA/H (K/D-region different) γ-irradiated spleen cells respectively in 1 ml cultures as described in Section 7.2.2. On day 5 the cultures were harvested, counted, and assayed for cytotoxic activity against ^{51}Cr-labelled P815 in the presence of Con A (10 μg/ml). Figure 7.1 shows the results of one such

Figure 7.1 Cytotoxic cell responses to I-, K/D-, or M-region controlled antigens. A.TL lymph node cells were mixed with γ-irradiated A.TH (●——●) or CBA/H (▲——▲) spleen cells, or BALB/c lymph node cells were mixed with γ-irradiated DBA/2 (■----■) or C57Bl (●——●) spleen cells in 1 ml cultures (Section 7.2.2). On day 5 cultures were assayed for cytotoxic activity as described in Section 7.2.2.


60
50
40
30
20
10
% SPECIFIC LYSIS
4.0
2.0
1.0
0.5
0.25
0.13
0.063
EFFECTOR : TARGET RATIO

experiment. The cultures stimulated through I-region (A.TL anti-A.TH) and through K/D-region antigens (A.TL anti-CBA/H) expressed equal levels of cytotoxic activity (solid curve). This effect was found to be highly reproducible over a series of four experiments. Treatment with anti-Thy 1.2 serum and complement showed cells in both these systems to be at least 98% T cells (Table 7.1).

Hence, both I-region and K/D region coded antigens stimulate strong and equivalent cytotoxic T cell responses in MLC when tested using a Con A-dependent lysis assay system. These data conflict with reports of others that stimulation through I-region coded determinants generates only weak cytotoxic T cell responses. (Bach *et al.*, 1972, 1973, 1976; Alter *et al.*, 1973; Abbasi *et al.*, 1973; Schendel *et al.*, 1973; Wagner *et al.*, 1975).

7.3.2 *T cell response to M-region coded determinants.*

Our interpretation of the data described in Section 7.3.1 depends on the assumption that the Con A-dependent lysis assay detects only cytotoxic T cells and that Con A itself does not cause the lysis of target cells in the presence of activated lymphocytes. We found that a T cell tumour line, the C57Bl/6 lymphoma EL-4, and activated T cells generated in MLC against M-locus (Mls) determinants were not cytotoxic in this assay system.

Anti-Mls blasts were generated in cultures containing BALB/c lymph node cells and γ-irradiated stimulator cells from DBA/2 spleens. This combination has been reported to give

TABLE 7.1

Treatment of I-region and K/D region stimulated cells with anti-Thy 1.2 and complement

Treatment	A.TL Ln cells stimulated with	
	†A.TH	CBA/H
AKR ascites + C'	3.4×10^6	1.3×10^6
C'	3.6×10^6	1.3×10^6
*Anti-Thy 1.2 (1:2) + C'	1.5×10^4	2.9×10^4
(% kill)	99.5%	98%

† A.TL responding lymph node cells (10^6) were mixed with either A.TH (I-region different) or CBA/H (K/D-region different) γ-irradiated spleen cells ($10^{6.2}$) as described (Section 7.2.2). Results are expressed as viable cells/ml.

* Anti-Thy 1.2 treatment was carried out as described in Section 7.2.4.

Ln, lymph node; C', guinea pig complement.

strong T cell proliferative responses in MLC (Bevan and Cohn, 1975). BALB/c mice are H-2^d/Mls^b and DBA/2 are H-2^d/Mls^a (Festenstein, 1976). BALB/c anti-DBA/2 blasts were tested in the Con A-dependent cytotoxicity assay described in Section 7.2.2 and compared with the lytic activity of cells generated against I-region or K/D region-antigens (Figure 7.1). The anti-Mls blast cells demonstrated very little cytotoxic activity in this system although a strong blast cell response was observed (Table 7.2). BALB/c T cells activated to C57Bl/6 stimulator cells (H-2 different) in the same culture system generated a similar blast cell response (Table 7.2) and a strong cytotoxic response equivalent to that observed for T cell responses to H-2I or H-2K/D different cells (Figure 7.1). Hence, stimulation through Mls coded determinants produces strong proliferative but only weak cytotoxic T cell responses in MLC. The C57Bl/6 T lymphoma, EL-4, did not cause any detectable lysis of P815 in the Con A-dependent cytotoxicity assay system at an effector to target ratio of 10:1.

These observations are in agreement with those of others (Bevan and Cohn, 1975; Röllinghoff *et al.*, 1975) that both a variety of T cell tumour lines and normal T cells activated to Mls determinants were not cytotoxic in this assay system. Hence, the Con A-dependent cytotoxicity assay detects only activated T cells with cytotoxic potential.

7.3.3 *Clonal analysis of cellular responses to I- and K/D-region coded determinants.*

T cells with cytotoxic potential are generated in equal numbers to both I-region and K/D-region coded determinants in

TABLE 7.2

Generation of blast cells in mixed cell cultures.

†Stimulating Cells	Responding Cells	Blast cells/culture
A.TH	A.TL	0.7×10^6
CBA/H	A.TL	1.5×10^6
DBA/2	BALB/c	1.2×10^6
C57Bl/6	BALB/c	1.3×10^6
BALB/c	BALB/c	4×10^4

† Cultures were set up as described in Section 7.2.2. γ-irradiated spleen cells ($10^{6.2}$) were mixed with lymph node responding cells (10^6) in 1 ml F-15 culture medium. Results are expressed as viable blast cells per culture.

bulk cultures (Section 7.3.1). However, this does not preclude the possibility that I-region stimulation generates two distinct classes of T cells, cytotoxic cells as well as non-cytotoxic helper cells. To test this possibility we have measured the precursor frequencies of proliferative and cytotoxic cells in A.TL lymph node cells responding to either I-region or K/D-region coded antigens in a limiting dilution culture system. If two distinct classes of T cells are stimulated to I-region coded determinants then the frequency of proliferative clones should be greater than the frequency of cytotoxic clones assuming that all clones consist of proliferating activated T cells.

Limiting dilutions of A.TL lymph node cells were mixed with γ-irradiated A.TL (syngeneic), A.TH (I-region different) or CBA (H-2K/D-region different) spleen cells in the presence of 10% CS in flat-bottomed microtitre trays as described in Section 7.2.3. On day 7 individual wells were scored visually, using an inverted microscope, for clones of activated lymphocytes and tested for cytotoxic activity using the Con A-dependent cytotoxicity assay system described above. The results of this experiment are described in Figure 7.2. The frequencies of proliferative clones, as determined by visual examination, and cytotoxic clones to all stimulator cells were equivalent (Table 7.3A). Also, most wells with visible clones were cytotoxic and of the wells that lacked visible clones few showed cytotoxic activity. Hence, there was a high correlation between proliferative and cytotoxic clones and very few wells containing proliferating non-cytotoxic clones were detected. Activation was found to be antigen-dependent since clones were not activated by syngeneic (A.TL) spleen cells (Figure 7.2). Hence, CS does

Figure 7.2 Distribution of negative wells in cultures containing limiting numbers of A.TL lymph node cells responding to γ-irradiated A.TL (■----■), A.TH (●————●), or CBA/H (▲————▲) spleen cells (Section 7.2.3). Individual wells were examined visually for the presence of activated lymphocytes (panel A) and tested for cytotoxic activity (panel B) as described in Section 7.2.3.

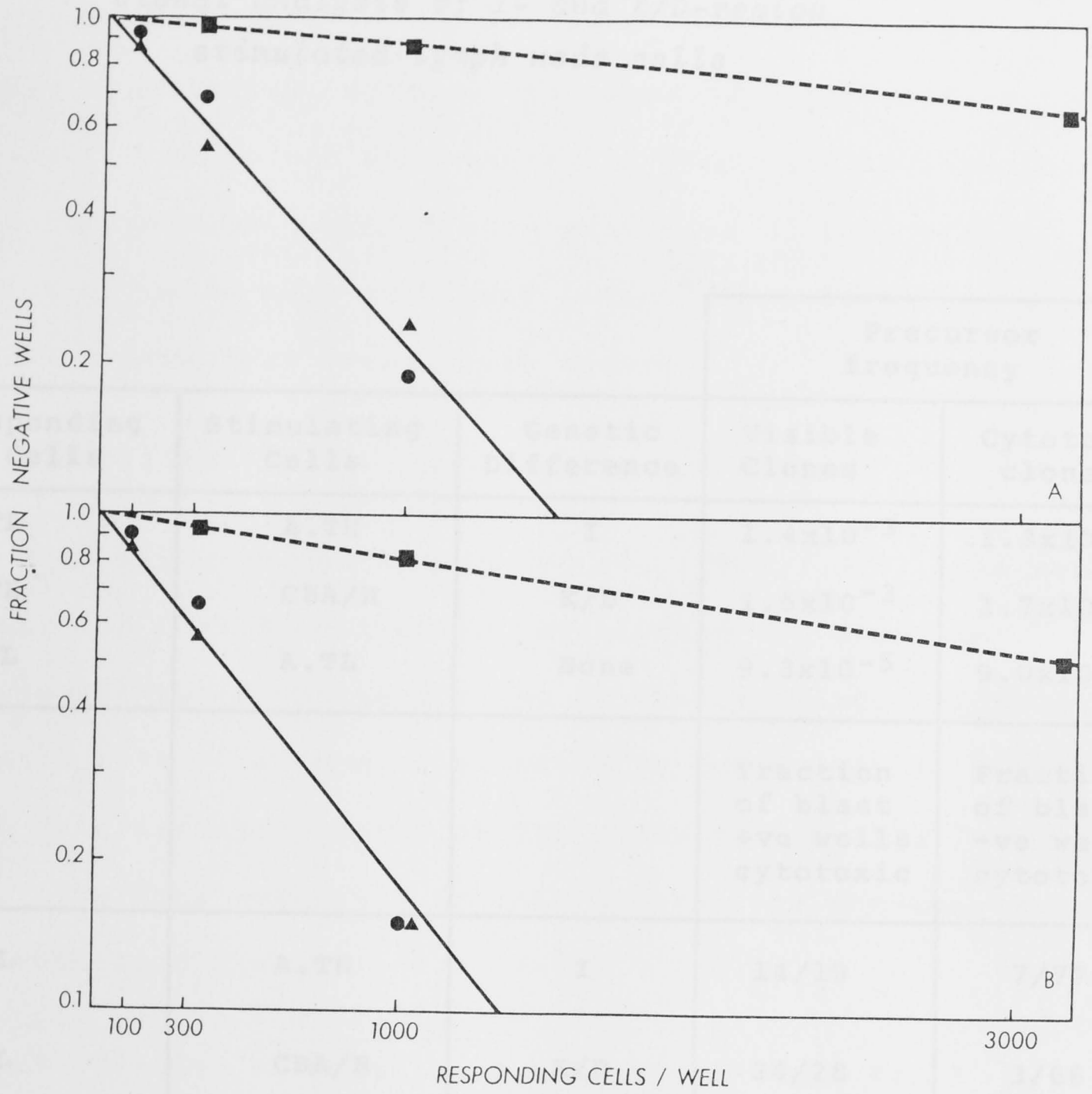

1.0
0.8
0.6
0.4
0.2
FRACTION NEGATIVE WELLS
A
1.0
0.8
0.6
0.4
0.2
0.1
B
100
300
1000
3000
RESPONDING CELLS / WELL

TABLE 7.3

Clonal analysis of I- and K/D-region stimulated lymph node cells

	Responding Cells	Stimulating Cells	Genetic Difference	Precursor frequency	
				Visible Clones	Cytotoxic clones
A	A.TL	A.TH	I	1.4×10^{-3}	1.3×10^{-3}
	A.TL	CBA/H	K/D	1.6×10^{-3}	1.7×10^{-3}
	A.TL	A.TL	None	9.3×10^{-5}	9.0×10^{-5}
				Fraction of blast +ve wells cytotoxic	Fraction of blast -ve wells cytotoxic
B	A.TL	A.TH	I	14/19	7/77
	A.TL	CBA/H	K/D	24/28	3/66

Cultures were set up as described in Section 7.2.3. γ-irradiated spleen cells ($10^{6.5}$/ml) were mixed with limiting dilutions of responding lymph node cells in 0.2 ml F-15 culture medium containing 10% CS. Individual wells were examined visually for activated lymphocytes and tested for cytotoxic activity as described (Section 7.2.3).

not cause the polyclonal activation of T cells in this system.

From these data we can conclude that, within the limits of this experimental system, no class of proliferating non-cytotoxic T cells can be detected in responses to I-region coded determinants. All clones activated in this system consist of cells with cytotoxic potential as detected using the Con A-dependent lysis assay system.

7.4 DISCUSSION

Bach and co-workers (Bach *et al.*, 1976, 1977) have proposed a model for T cell activation postulating the absolute requirement for a distinct class of non-cytotoxic helper T cells (T_H). T_H cells are activated predominantly to I-region coded determinants present on the stimulating cells (see Section 1.5). The data described in this chapter are not compatible with this hypothesis.

Stimulation of T cells in MLC through H-2I region differences generates cells with cytotoxic potential, equal in numbers to that seen with responses to H-2K/D controlled histo-compatibility antigens. This response generated in bulk cultures (Section 7.3.1) can be measured using a non-specific Con A-dependent lysis assay. That this assay detects only cells with cytotoxic potential is suggested by the fact that anti-Mls T cell blasts and EL-4 tumour cells do not lyse P815 under the same conditions. Bevan and Cohn (1975) have further shown that a number of other T lymphomas also do not lyse P815 in the presence of Con A.

Clonal analysis has shown that the precursor frequency for proliferative and cytotoxic clones to I-region determinants is the same (Section 7.3.3). Hence T cells with cytotoxic potential are the predominant if not the only class of T cell responsive to both H-2I and H-2K/D controlled determinants. Hence, there is no evidence for the existence of a distinct class of non-cytotoxic helper T cell responsive to I-region controlled determinants, within the limits of sensitivity of our experimental system. These observations are not compatible with the hypothesis proposed by Bach and co-workers. Their model predicts that I-region stimulation of T cells will generate predominantly clones of non-cytotoxic helper T cells.

We conclude from our data that the low levels of cytotoxic activity often reported in I-region stimulated MLC systems is not attributable to the absence of cytotoxic T cells in the culture. Rather, the lack of cytotoxic activity must be attributable to the use of target cells inappropriate for the detection of specific cytotoxic responses directed against I-region controlled determinants. This is not unlikely considering the restricted distribution of Ia antigens as compared with K/D-controlled histocompatibility antigens (Shreffler and David, 1975). Wagner *et al.* (1975) found that PHA-stimulated blast cells, the target cells most commonly used in these studies, were particularly poor for detecting cytotoxic responses against I-region controlled determinants. In this light it may, in fact, be never possible to find a target cell population as sensitive to killing through I-region controlled determinants as through K/D-controlled histocompatibility antigens.

In conclusion, both H-2K/D and H-2I region controlled determinants stimulate, predominantly, cytotoxic T cell responses *in vitro* when present on S^+ stimulator cells and no collaboration between the two is required. This conclusion is in agreement with the views recently expounded by Klein (1978). Cantor and Boyse (1975b) showed that there is no requirement for the putative class of Ly-1^+ helper T cells (T_H) under optimal conditions of stimulation in MLC. Also, Miller *et al.* (1977) reported that, in a limit dilution system containing athymic (nude) filler spleen cells, no further responding cell was limiting other than the cytotoxic T cell (T_C) precursor. They have concluded from these data that there is no requirement for the collaboration of distinct classes of responding cells in this system. Hence, T_C cell activation does not require a distinct class of non-cytotoxic helper T cell as proposed by Bach *et al* (1976, 1977). The synergistic effects sometimes observed between different populations of responding cells under suboptimal culture conditions (Wagner, 1973; Cantor and Boyse, 1975b) do not reflect such a requirement. It is probable that all activated cells, including T_C cells, have the capacity to 'help' by releasing lymphokines into the culture medium. Hence, populations of T cells do appear to collaborate in these *in vitro* systems in a synergistic fashion.

7.5 SUMMARY

In vitro responses to I-region incompatible stimulator cells do not generate selectively clones of a distinct class of non-cytotoxic helper T cell. Most clones generated in these responses have cytotoxic potential which can be detected with a non-

specific Con A dependent lysis assay system. These observations are not compatible with the T_C/T_H collaboration model proposed by Bach and co-workers.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

In this Chapter I will deal first with the observations characteristic of T cell responses to histocompatibility antigens *in vitro*. In the second section I will describe a theoretical model for T cell activation which, at this stage, can account for these observed phenomena.

8.1 CHARACTERISTICS OF T CELL ACTIVATION *IN VITRO* - AN EMPIRICAL ANALYSIS.

The generation of T cell responses *in vitro* has been the subject of many conflicting reports in the literature over the last decade. Particularly controversial have been such aspects of T cell activation as responsiveness to dead cells, subcellular fractions and xenogeneic leucocytes, as well as to cells from congenic mice which differ from the responding cells at single loci within the MHC. Yet the construction of an accurate theoretical model of T cell activation in such systems relies on the accuracy and interpretation of these basic observations. Hence, the well-accepted T_H/T_C collaboration model of T cell activation proposed by Bach and co-workers (1976, 1977) was based on a series of observations whose accuracy and interpretation have recently been thrown into considerable doubt (Klein, 1978; Chapter 7). We believe that a number of characteristic properties of *in vitro* T cell responses can now be recognized. Based on these observations a simple two cell interaction model of T cell activation can be postulated (Section 8.2).

According to a number of studies, histocompatibility antigens on dead cells, membrane fragments, and on many classes of non-lymphoid cells do not stimulate in mixed cell cultures. In our system the DBA/2 mammary carcinoma CaD2 does not stimulate a

detectable cytotoxic T cell response in allogeneic C57Bl/6 lymph node cells. The DBA/2 mastocytoma P815, on the other hand, stimulates strong cytotoxic T cell responses in the same culture system (Section 4.3.1). Hence, cell populations can be classified as stimulatory (S^+) or non-stimulatory (S^-) according to their capacity to stimulate appropriate T cell responses in mixed cell cultures following γ-irradiation. Treatment of the (S^+) tumour P815 with UV-radiation renders it non-stimulatory (S^-); the S^+ phenotype is destroyed by UV-radiation (Section 4.3.2).

Both the UV-irradiated P815 and γ-irradiated CaD2 S^- tumours are fully stimulatory in the presence of a Con A-stimulated cell supernatant (CS). This demonstrates that both UV-irradiated P815 and γ-irradiated CaD2 carry appropriate antigenic determinants for the stimulation of full and specific cytotoxic T cell responses in the presence of CS, suggesting that the defect in these S^- stimulator populations is not at the level of antigen presentation. It is possible that UV-irradiated stimulator cells, in contrast to γ-irradiated P815, are unable to replenish antigens shed during culture. Our observations that UV-irradiated P815 are non-stimulatory at densities two orders of magnitude greater than sufficient for T cell activation by γ-irradiated P815 (Section 4.3.3) suggest that this does not account for the S^- phenotype of UV-irradiated P815. The intrinsically S^- tumour CaD2 also may express less histocompatibility antigens on its surface than P815 but, in the presence of CS, it is also a highly efficient stimulator of H-2^d reactive cytotoxic T cells.

Our observations that antigen alone is non-immunogenic in mixed cell cultures are by no means unanimously supported by other reports in the literature. Wagner and Röllinghoff (1976) reported that even heavy doses of UV-radiation abrogated but did not completely destroy the stimulatory capacity of spleen cells in MLC. Our own studies (Talmage *et al.*, 1977) indicated that small responses to UV-irradiated P815 were occasionally observed, but never in our own laboratory at the Australian National University in Canberra. Lemonnier *et al* (1978) reported a small but significant cytotoxic response to purified plasma membranes in contrast to other reports claiming that no response to either membrane fragments or soluble histocompatibility antigens is observed in culture (reviewed in Section 1.4).

Our studies suggest that any agent which non-specifically activates leucocytes could, by doing so, stimulate the release of CS activity. Hence, any mitogenic effect of foetal calf serum and/or 2-mercaptoethanol may initiate the release of CS activity from the responding cells in the culture. Under these conditions, responses to UV-irradiated cells and subcellular fractions may occur. Such mitogenic effects of foetal calf serum and 2-ME have been described (Byrd, 1971; Lemke and Opitz, 1976; Goodman and Weigle, 1977).

From these observations we conclude that there are at least two distinct requirements for the activation of cytotoxic T cells to foreign histocompatibility antigens *in vitro* - antigen and a soluble, cell-derived factor present in CS but normally provided endogenously by the metabolically intact stimulating cells. The

fact that UV-radiation completely destroys the stimulatory capacity of P815 and that CS is required within the first day of culture suggests that the active factors in CS are required during the early inductive phase of the response.

Studies involving the proliferation of cytotoxic T cells in long term culture demonstrated at least two separable activities in CS, both being required for the ultimate expression of full cytotoxic T cell responses *in vitro*. Early studies (Section 4.3.4) indicated that CS was required continuously throughout the standard five day culture period. This phenomenon was further investigated in studies described in Chapter 6. The continued proliferation of cytotoxic T cells in long term culture was found to be dependent on a soluble activity, present in CS, which we have called 'maintenance' factor. This factor did not share the species specificity of CS activity required for the activation of cytotoxic T cells to UV-irradiated P815 (Chapter 5). Hence, two separable activities required for cytotoxic T cell activation *in vitro* are present in CS. One activity acts on mature proliferating T cells and is, essentially, not species specific. This we have called 'maintenance' activity. A further activity, which we have called the 'lymphocyte costimulator' (Paetkau *et al.*, 1976) is species specific (Chapter 5) and is not required for the proliferation of mature T_C cells. Costimulator acts early during the response and is required to drive the T_C precursor cells to the stage where they are responsive to 'maintenance' activity. It is, hence, inductive in nature. The 'maintenance' activity will not support the early inductive phase of T_C activation (Section 6.3.3).

In our system both mixed lymphocyte and cytotoxic cell responses are phylogenetically restricted (Chapter 5). This restriction can be completely overcome by the use of CS preparations homologous, but not heterologous, with the responding cells. CS derived from guinea pig, bovine, or human leucocytes cannot restore the response of C57Bl/6 lymph node cells to the UV-irradiated allogeneic tumour P815. Each preparation can, however, support the response of homologous leucocytes to γ-irradiated P815 in xenogeneic cultures. Hence, CS activity is specific for the species of the responding cells.

Subsequent experiments using a limit dilution assay system demonstrated that although guinea pig lymph node cells do not respond to γ-irradiated P815 in the absence of CS they contain a high frequency of P815-reactive precursor cytotoxic cells equal to that found in lymph node populations from allogeneic mouse strains. Hence, the lack of response of guinea pig leucocytes to the xenogeneic P815 tumour is not attributable to a low precursor frequency. Lafferty and Cunningham (1975) suggested that the lack of responsiveness between xenogeneic leucocytes was a reflection of the species specificity of the inductive 'signal 2' required for leucocyte activation. The data described in Chapter 5 are in accordance with this hypothesis. We propose that the phylogenetic restriction of cytotoxic T cell responses is due to the species specificity of the lymphocyte costimulator which is absolutely required for T cell activation.

This hypothesis predicts that responses to xenogeneic cells will occur if and only if costimulator homologous with the

responding cells is present in the culture system. As was suggested above costimulator may be generated from responding spleen cells in the presence of mitogenic batches of foetal calf serum and 2-ME. Under these conditions xenogeneic responses could occur in the absence of exogenous CS. This may account for a number of reports of responses in xenogeneic MLC systems since batches of foetal calf serum are often selected for their capacity to support strong mixed lymphocyte responses.

One characteristic of T cell responses *in vitro* which has been particularly controversial involves the putative role of helper T cells in the generation of cytotoxic T cells to allogeneic histocompatibility antigens. Bach and co-workers (1976, 1977) have proposed a model of cytotoxic T cell activation which postulates that a distinct class of non-cytotoxic helper T cells, stimulated predominantly by I-region-coded Class II antigens[1], provide an essential inductive signal (signal 2) to the precursor cytotoxic T cells, either directly or via soluble factors. This hypothesis has gained support from the work of Cantor and Boyse (1975a, 1975b, 1976, 1977) who proposed that T cell subpopulations can be defined according to the Ly surface antigen system, and that this classification system can be correlated with the functional classification proposed by Bach. Hence, Ly-1$^+$ helper T cells (T_H) are stimulated by Class II antigens while Ly-2,3$^+$ cytotoxic T cells (T_C) are stimulated by Class I antigens.

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1 For convenience we have adopted the nomenclature for MHC-coded antigens described by Klein (1977). By this system the H-2K and H-2D regions code for Class I antigens and the H-2I region codes for Class II antigens.

However, Cantor and Boyse (1975b) found that Ly-1$^+$ cells were not essential for the activation of Ly-2,3$^+$ cytotoxic cells to Class I antigens under optimal culture conditions. This observation has been supported by a number of other studies. Miller *et al* (1977) found that in a limit dilution assay system in the presence of excess athymic (nude) spleen filler cells only the cytotoxic precursor cells were limiting. Furthermore, strong cytotoxic T cell responses occur even in combinations in which the responders differ from the stimulators (and targets) by a single mutation within one locus of the H-2K or H-2D region (Berke and Amos, 1973; Forman and Klein, 1975; Nabholz *et al.*, 1975; Melief *et al.*, 1977). Also, there is considerable evidence that Class I and Class II antigens do not stimulate functionally distinct classes of T cells. Specific cytotoxic responses are easily generated to Class II antigens in the absence of Class I antigenic differences (Klein *et al.*, 1977; Wagner *et al.*, 1975; Davidson, 1977). In fact both Class I and Class II antigens stimulate predominantly clones of cytotoxic T cells in culture (Chapter 7). Taken together, these observations negate the hypothesis that two distinct classes of T cells are required for the generation of cytotoxic T cell responses. This does not preclude the possibility that activated cells of any type - including cytotoxic T cells - can 'help' under suboptimal culture conditions. Preliminary experiments carried out in our own laboratory indicate that activated cytotoxic T cells stimulated by either Class I or Class II antigens can produce CS activity in the presence of Con A (Andrus, personal communication). What Ly phenotype is expressed by Class II antigen-stimulated cytotoxic T cells has not been determined.

Accessory cells of various types, in particular cells of the macrophage-monocyte class, have been implicated as playing an essential role in the induction of immune responses *in vitro*, including B cell responses (Mishell and Miller, 1975; Rosenstreich and Oppenheim, 1976) and T cell responses to both mitogens (Rosenstreich *et al.*, 1976; Mookerjee, 1977) and allogeneic antigen (Schilling *et al.*, 1976; Wagner *et al.*, 1972; Mishell and Miller, 1975; Gorczynski, 1976; Davidson, 1977). A number of studies have demonstrated that accessory cell function could be provided by the addition of 2-ME to the culture medium (Lemke and Opitz, 1976; Koren and Hodes, 1977). These observations exemplify the fact that many *in vitro* requirements for immune cell activation may merely reflect deficiencies in the culture system. A number of workers (Rosenstreich and Oppenheim, 1976; Davidson, 1977) have reported a requirement for accessory cells for T cell responses *in vitro* in the presence of 2-ME but these too may reflect deficiencies of the culture system, which although very important for the development of more sophisticated culture techniques may not be relevant immunologically.

The studies described in Section 4.3.3 demonstrated that nylon wool column purified T cells exhibit an unimpaired response to the allogeneic tumour P815 in the absence of added factors. This indicates that a special class of adherent accessory cells is not required for the generation of cytotoxic T cell responses *in vitro*. Similarly, Davidson (1977) found that depletion of adherent cells with carbonyl iron did not impair the cytotoxic response of lymph node cells to allogeneic spleen cell stimulators. Depletion of the

stimulating spleen cells in the same way, however, caused a marked abrogation of the response. This does not necessarily indicate a requirement for stimulator-type accessory cells since carbonyl iron treatment may cause a depletion of S^+ stimulator cells from the spleen cell population.

Hence we conclude that only two cells are required for T cell activation - the responding T cell and the metabolically intact S^+ stimulator cell. On this basis a simple theoretical model for cytotoxic T cell activation can be formulated.

8.2 A THEORETICAL TWO CELL INTERACTION MODEL OF CYTOTOXIC T.CELL INDUCTION.

We have proposed a model of T cell activation which is compatible with the phenomena discussed above. This model is based on two simple postulates.

1. Antigen alone is non-immunogenic for cytotoxic T cell precursors. Stimulation is a function of specific classes of S^+ haemopoietic cells such as macrophages and activated lymphocytes which provide an inductive second signal, the lymphocyte costimulator, to the responding cell.

2. T cell activation involves only two classes of cells, the S^+ stimulating cell and the responding T cell. There is no requirement for the interaction of more than one class of responding cell.

Hence, cytotoxic T cell activation *in vitro* is a two signal event and both signals can be provided by the S^+ stimulating cell. We propose that the second signal is an inductive factor,

which we have called the lymphocyte 'costimulator'. This factor is normally provided by the stimulating cell but is also present in a Con A-stimulated cell supernatant (CS). According to this model neither antigen nor costimulator alone are stimulatory. This is borne out by the observation that CS is not a polyclonal activator of T cells in syngeneic cell cultures (Section 7.3.3).

Normal induction of cytotoxic T cells occurs when antigen-reactive T cell precursors interact with antigen on the surface of syngeneic S^+ cells which can provide the inductive second signal (costimulator) (Figure 8.1A). Cytotoxic T cell responses to chemically- (Shearer *et al.*, 1975; Forman, 1975) or viral-modified syngeneic cells (Zinkernagel and Doherty, 1975; Zinkernagel *et al.*, 1976) are generally restricted to the MHC phenotype of the stimulating cell. This phenomenon, called H-2 restriction with respect to the murine system, suggests that products of the major histocompatibility complex (MHC) are intimately involved in the activation of cytotoxic T cells. We propose that interaction between antigen and MHC products on the cell surface are essential for the ultimate release of costimulator from the syngeneic S^+ cells and the generation of cytotoxic T cell responses. Antigens which do not interact with H-2 products of a particular genotype will be unable to stimulate the release of costimulator from S^+ stimulating cells carrying that phenotype. For this reason such genes will behave as immune response (Ir) genes for that antigen. This postulate is compatible with the observations that Ir genes for a variety of T cell responses map predominantly to the MHC (Miller *et al.*, 1976; Green, 1974; Meruelo *et al.*,

Figure 8.1 Interactions occurring between different populations of stimulator and responding cells predicted by the proposed 'two signal' mechanism for T cell activation.

(A) Cells responding to foreign antigen (signal 1) on autologous stimulator cells which provide signal 2. Activation occurs.

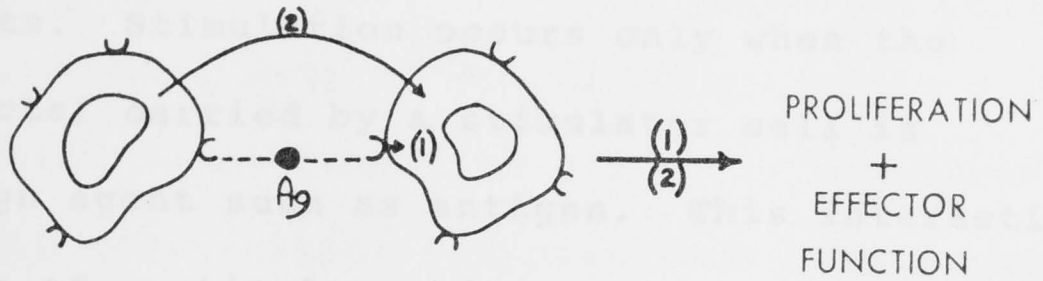
(B) Cells responding to histocompatibility antigen (signal 1) on allogeneic stimulator cells which provide signal 2. Activation occurs.

(C) Cells interacting with UV-irradiated allogeneic stimulator cells. Signal 1 (antigen) is presented but no signal 2 is provided by the stimulating cell. Activation does not occur because no signal 2 is provided to the responding cell.

(D) Cells interacting with xenogeneic stimulator cells. Signal 1 (antigen) and signal 2 are both provided by the stimulator cell. Activation does not occur because the responding cell cannot recognize the xenogeneic form of signal 2.

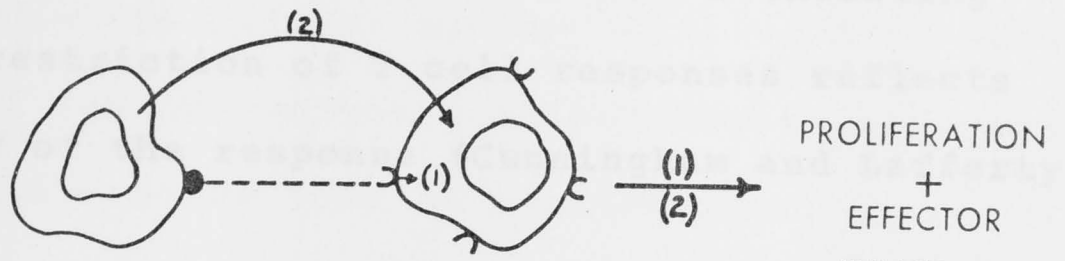
Stimulator Responder

A. NORMAL ANTIGENIC INDUCTION



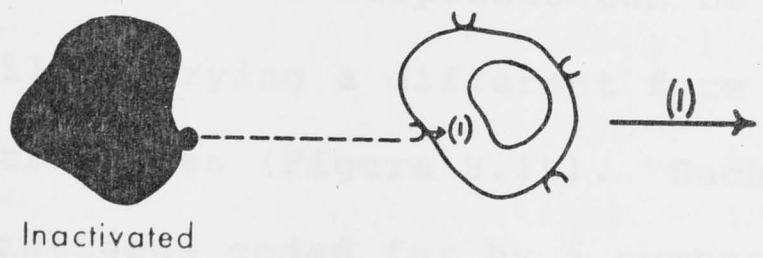
PROLIFERATION + EFFECTOR FUNCTION

B. ALLOGENEIC STIMULATION (Abnormal Induction)



PROLIFERATION + EFFECTOR FUNCTION

C. ALLOGENEIC INTERACTION (No Signal '2')



Inactivated

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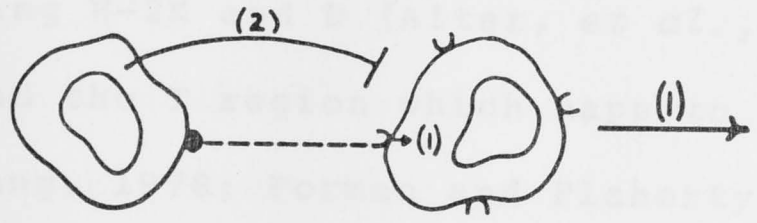
D. XENOGENEIC INTERACTION

(Signal '2' not recognized)



-ve

1977) and that non-responsiveness in some systems is caused by a defect at the level of antigen presentation by the stimulating (S^+) macrophages (Shevach and Rosenthal, 1973; Shevach, 1976). In this way, products of the MHC act as 'self-markers' which forbid self-stimulation by autologous S^+ cells. The lymphocyte costimulator is not released from S^+ cells carrying an unaltered form of the MHC products. Stimulation occurs only when the self-marker (MHC products) carried by a stimulator cell is modified by some foreign agent such as antigen. This interaction then causes the release of costimulator from the stimulating cell. Hence the H-2 restriction of T cell responses reflects the activation history of the response (Cunningham and Lafferty, 1977; Shevach, 1978).

Unrestricted cytotoxic T cell responses can be generated only to stimulating cells carrying a different form of the self/not self recognition structures (Figure 8.1B). Such responses have been observed to antigens coded for by a number of distinct genetic regions including H-2K and D (Alter, *et al.*, 1973), H-2I (Klein *et al.*, 1977) and the T region which maps to the right of H-2D (Klein and Chiang, 1978; Forman and Flaherty, 1978). Interaction between histoincompatible cells in these situations causes the release of costimulator from the stimulating cells because the self markers themselves are also acting as antigen. On this basis H-2K/D, H-2I and T region-coded products should all have the capacity to serve independently as self-recognition structures and antigen interaction with any of these structures should be sufficient to cause T cell activation. The apparent restriction of many cytotoxic T cell responses to H-2K and H-2D regions of the MHC may merely reflect the more efficient killing

often observed through Class I antigens than through Class II antigens (Davidson, 1977; Chapter 7).

The phylogenetic restriction of mixed lymphocyte and cytotoxic T cell responses is wholly attributable to the species specificity of costimulator (Figure 8.1D). This postulate is supported by the observation that guinea pig lymph node cells express a high frequency of precursor cytotoxic cells reactive to the xenogeneic mouse tumour P815 but are unresponsive to this cell line unless costimulator prepared from guinea pig spleen cells is added to the culture medium (Chapter 5). Guinea pig lymphocytes cannot respond to mouse stimulator cells because they cannot recognize the mouse form of costimulator.

Metabolic inhibition of the stimulating cells destroys their capacity to produce and/or release costimulator hence rendering them S^- (Figure 8.1C). Responses to S^- cells are generated in the presence of CS which contains a soluble form of costimulator.

A further soluble activity influencing cytotoxic T cell responses *in vitro* has been described in Chapter 4. Maintenance activity, also present in CS, can support the continued proliferation of cytotoxic T cells in culture but cannot replace a requirement for costimulator. This activity may or may not have immunological relevance and has not been included in the theoretical model described above.

As with all *in vitro* studies the system described in this thesis can give only an indication of the physiological system as it functions *in vivo*. There are, however, a number of

similarities between the responses of T cells *in vitro* and *in vivo*. These have been discussed in Chapter 1 (Sections 1.3 and 1.4). In general, responses occur only to allogeneic viable cells of the lympho-myeloid class, both *in vitro* and *in vivo*. On this basis we propose that costimulator plays an important physiological role in the activation of T cell responses.

Our model for T cell activation is compatible with generally held concepts of haemopoiesis. According to this model pluripotential stem cells seed from the bone marrow to primary haemopoietic organs such as the spleen. Here, under the influence of haemopoietic inductive microenvironments (Curry and Trentin, 1967) a proportion of stem cells become committed to a particular differentiation pathway. Hence, stem cells in the red pulp areas of the spleen are generally committed to differentiate along the erythropoietic cell lineage. In this respect the thymus exerts a similar effect on stem cells committing them to the T cell lineage. According to the general model of haemopoiesis committed progenitor cells are induced to differentiate and proliferate both *in vivo* and *in vitro* under the influence of humoral factors. This has been shown for the formation of erythroid (Stephenson *et al.*, 1971) megakaryocytic (Nakeff, 1977) and myeloid (Metcalf and Moore, 1973) cell series. Our model proposes a similar mechanism for the activation of T cells. Under the influence of costimulator committed antigen specific T progenitor cells are stimulated to differentiate and proliferate to form mature cytotoxic effector cells. The major difference between this system and that controlling the generation of other classes of haemopoietic cells is the

specificity of the response. An additional control mechanism has been superimposed on the pre-existing haemopoietic system. The responding T progenitor cell must now engage antigen to be receptive to the inductive costimulator stimulus. In this way a non-specific inductive system has evolved to form an adaptive immune system. For a better understanding of these complex adaptive immune systems it may then be valuable to examine more closely the non-specific inflammatory systems of vertebrate immunity. Like the specific immunocytes, which were the subject of this thesis, non-immunocompetent elements of the haemopoietic system also display a strong reactivity to allogeneic and closely related but not to unrelated xenogeneic blood cells in graft versus host (GvH) type reactions (Payne and Jaffe, 1962; Lafferty *et al.*, 1972). It would appear that the MHC serves as the self/not self recognition structure for the more primitive non-specific inflammatory system as well as for the more sophisticated adaptive immune system.

Similarly, certain invertebrate immune systems appear to function along similar lines. The colonial ascidian has what appears to be a primitive version of the MHC of vertebrates which controls the interaction of haemolymph cells (Oka and Watanabe, 1957; Mukai, 1967; Tanaka, 1975).

On this basis a useful approach to the study of specific immunity in vertebrates may be to consider it in the light of these more primitive reactions rather than as a system peculiar to vertebrates and wholly divorced from other elements of the haemopoietic system for the activation of both specific immunocytes and the non-specific inflammatory blood cells would

appear to be controlled by the same fundamental mechanism. Antigen specificity merely represents a 'fine-tuning' of a pre-existing self/not self recognition system controlled through the Major Histocompatibility System.

APPENDIX ONE

This section describes the derivation of the statistical model commonly used to estimate the frequency of antigen-specific precursor immunocytes. This model describes the distribution of non-responsive wells when varying numbers 'n' of responding cells are randomly distributed in individual wells of micro-titre plates, together with the appropriate stimulating antigen, for any given precursor frequency 'p'. This model has been used extensively to estimate the precursor frequency of antibody-producing B cells (Quintáns and Lefkovits, 1973) and of cytotoxic T cells (Lindahl and Wilson, 1977; Teh *et al.*, 1977).

By a direct application of the Binomial theory (Moroney, 1951) the probabilities of 0, 1, 2, 3, 4, etc., precursors in a sample population of 'n' cells distributed at random from a large population of cells whose proportion of precursors is 'p', are given by the successive terms of the expansion of the binomial expression

$$(q + p)^n + q^n + n.q^{n-1}.p + \text{---} + n.q.p^{n-1} + p^n \qquad [1]$$

reading from left to right, where $q = 1 - p$.

Hence,

$$P(0) = q^n;\ P(1) = n.q^{n-1}.p;\ \text{etc.},$$

where P(0) equals the expected frequency of wells containing no precursors, P(1) equals the expected frequency of wells containing a single precursor, etc.

Now, in cases where either 'p' or 'q' are very small (e.g. where $p < 0.05$) the Binomial distribution can be approximated

very closely using the Poisson distribution

$$1 = e^{-z} + z.e^{-z} + \frac{z^2}{2!}.e^{-z} + \text{---} \qquad [2]$$

where 'z' equals the average number of precursors per sample. In this case 'z' equals the product of the precursor frequency 'p' and the sample size 'n'

$$z = n.p. \qquad [3]$$

Hence, for small values of 'p', and substituting equation [3],

$$P(0) = q^n \sim e^{-n.p.}$$

$$P(1) = n.q^{n-1}.p \sim n.pe^{-n.p}$$

etc.

From this model we can see that in systems where the progeny of a *single* precursor can be detected, the expected frequency of negative wells [P(neg)] is defined by the equation

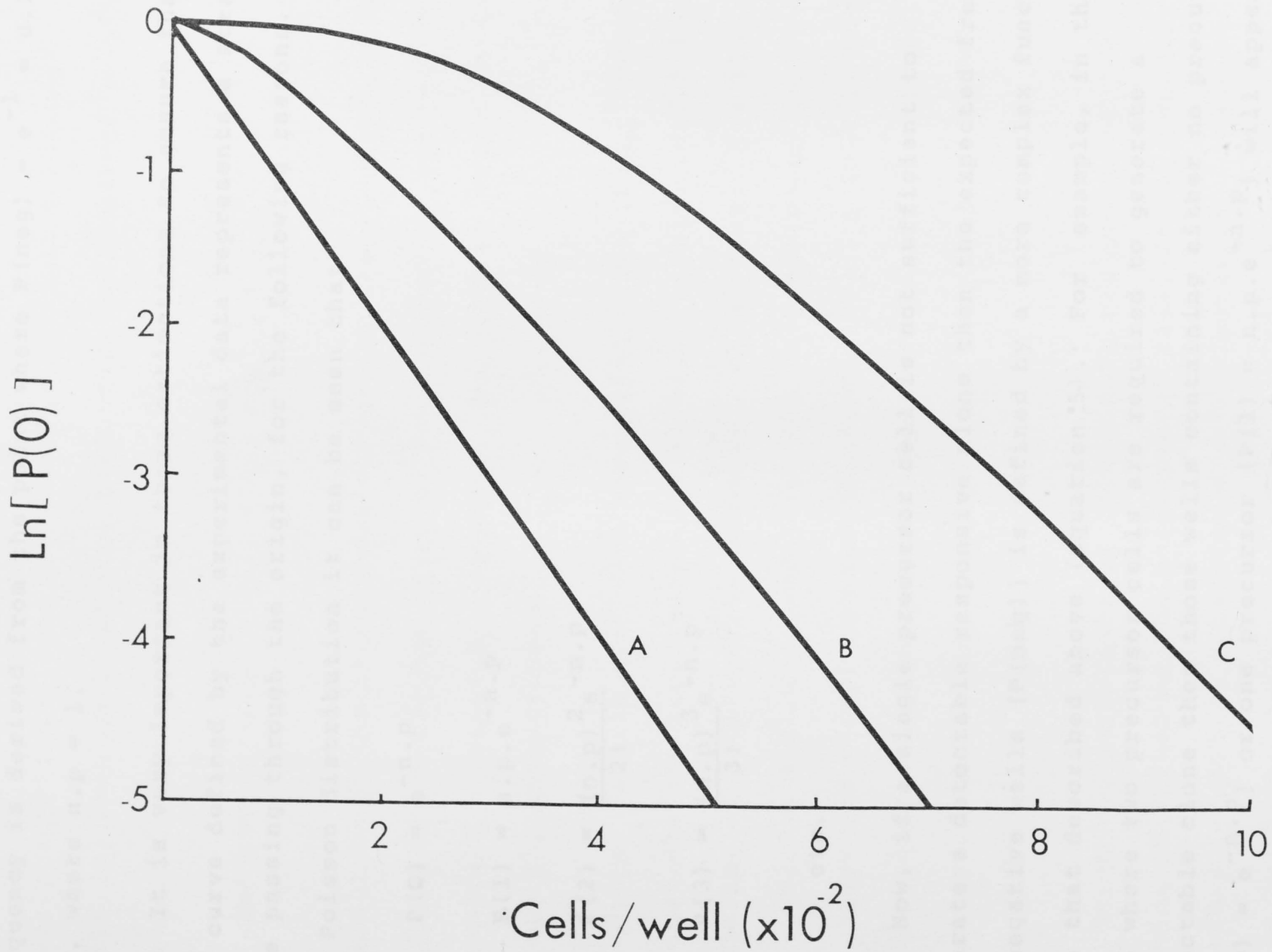
$$P(neg) = P(0) = e^{-n.p} \qquad [4]$$

$$\therefore \quad LnP(neg) = -n.p \qquad [5]$$

Hence, the frequency of non-responsive wells is described by the function (equation 5) which describes a straight line, passing through the origin (when n = 0, LnP(neg) = 0) with slope '-p', which defines the precursor frequency.

Figure 1 (curve A) shows a theoretical example based on this model. The precursor frequency 'p' can be derived from the point at which LnP(neg) = -1, ($P(neg) = e^{-1}$), i.e. where n.p (the average number of precursors per well) = 1. This

Figure A.1 Theoretical distribution of negative wells containing limiting numbers of responding cells with a precursor frequency of 10^{-2}, according to Poisson statistics, where A) 1, B) 2, or C) 4 precursor cells are required to generate a detectable response. P(O) represents the proportion of negative wells for each given dose of responding cells.


0
-1
-2
-3
-4
-5
Ln[P(0)]
A
B
C
2
4
6
8
10
Cells/well (x10^-2)

curve is generally plotted as the function $P(neg) = e^{-n.p}$ using semi-logarithmic graph paper. Here again the precursor frequency is derived from the point where $P(neg) = e^{-1} = 0.37$, i.e. where $n.p = 1$.

It is very important in these estimations to ensure that the curve defined by the experimental data represents a straight line passing through the origin, for the following reasons. From the Poisson distribution it can be seen that

$$P(0) = e^{-n.p}$$

$$P(1) = n.p.e^{-n.p}$$

$$P(2) = \frac{(n.p)^2 e^{-n.p}}{2!}$$

$$P(3) = \frac{(n.p)^3 e^{-n.p}}{3!}$$

etc.

Now, if a single precursor cell is not sufficient to generate a detectable responsive clone then the expected frequency of negative wells [P(neg)] is defined by a more complex function than that described above (equation 5). For example, in the case where two precursor cells are required to generate a detectable clone then those wells containing either no precursor [$P(0) = e^{-n.p}$] or one precursor [$P(1) = n.p.e^{-n.p}$] will appear negative.

Hence,

$$P(neg) = P(0) + P(1)$$

$$= e^{-n.p} + n.p.e^{-n.p}$$

$$= e^{-n.p} (1 + n.p).$$

$$\therefore \quad \text{Ln } P(\text{neg}) = -n.p + \text{Ln } (1 + n.p) \qquad [6]$$

Clearly, equation [6]does not describe a linear function. Similarly, when at least four precursors are required to give a detectable positive,

$$\text{LnP(neg)} = -n.p + \text{Ln}(1 + n.p + \frac{n^2p^2}{2} + \frac{n^3p^3}{6}) \qquad [7]$$

Figure A1 shows the theoretical curves for the situation where p = 0.01, and where one, two, or four precursors are required to generate a detectable response, described by equations [5], [6] and [7] respectively.

Hence, experimental data describing a non-linear function may indicate the requirement for multiple precursors for the generation of a detectable response. A non-linear function can also indicate the requirement for the interaction of different cell types for clonal expression (Groves *et al.*, 1970). For these reasons, only experimental data describing the appropriate linear function have been used for the estimation of precursor frequencies.

Given the importance of determining the nature of the function described by the experimental data, it is obviously essential to include data over a sufficiently wide range of values for P(neg) to be reasonably sure that the data cannot be fitted to a curve. In the studies described in this thesis values for P(neg) ranging from 0.1 to 1.0 have been used wherever possible.

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